GRAHAM LUSK[1]

(February 15, 1866 – July 18, 1932)

Graham Lusk has become a traditional figure in American science. During the 18 years which have elapsed since his death there have been rapid advances in the science of nutrition in the United States. Many of our present workers did not have the privilege of knowing him and thus of appreciating in a personal way the scientific contributions which he made and the leadership which he gave. Professor Lusk was one of the original group which organized the American Institute of Nutrition in 1928 for the corporate support of the new Journal of Nutrition. He served on the editorial board of the Journal until his death on July 18, 1932.[2] A picture of Graham Lusk appears on the front cover of this number and will appear on subsequent issues of volume 41 of THE JOURNAL OF NUTRITION. He is the first of the modern American workers in nutrition to be thus honored.

Graham Lusk was born in Bridgeport, Connecticut, on February 15, 1866. He was the son of a distinguished physician, William Thompson Lusk, who was an obstetrician of note and the writer (in 1882) of an authoritative treatise in that field. His father had hoped that Graham would become a physician; however, his impaired hearing precluded such a

[1] Several excellent biographies have appeared earlier. These include one by J. R. Murlin (J. Nutrition, *5:* 527–538, 1932; another by G. B. Wallace on his relation to the Harvey Society (The Harvey Lectures, 1931–1932, 5–8); and a more recent one by Eugene F. DuBois, National Academy of Sciences, vol. 21, Third Memoir, 1940.

[2] Younger members of the American Institute of Nutrition may not be aware that this society assumed its present form as a professional society of research nutritionists on April 11, 1933, was incorporated as such November 16, 1934, and was admitted to the Federation of American Societies for Experimental Biology in 1940.

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calling and his interest was to be centered in physiological chemistry. Even though he did not receive formal medical training, Lusk never lost his interest in the metabolic aspects of medicine. He probably accomplished more for the advancement of our knowledge in medicine by his physiological chemical approach than would have been possible from a clinical attack on such problems.

His basic training in chemistry was obtained at the Columbia University School of Mines, from which he was graduated with a Ph.B. degree in 1887. Lusk spent the next 4 years in Germany. Before he was accepted for graduate work, it was first necessary for him to devote a year to gaining proficiency in the language. After that he spent three years in graduate training, both at Leipzig under Carl Ludwig and at Munich with Carl Voit. It was with the latter master that he gained the enthusiasm which he carried throughout his life. Lusk received the Ph.D. degree in 1891 from the University of Munich.

After his return to the United States, Lusk became an instructor of physiology at the Yale Medical School. In this first position he served not only in an instructional capacity but he was also his own janitor at the munificent salary of $30 per month! The ability with which he served his apprenticeship is attested to by the fact that he became an Assistant Professor the following year and Professor of Physiology three years later, in 1895. Graham Lusk accepted the professorship in physiology at the University and Bellevue Hospital Medical College in New York City in 1898. His father had been Professor of Obstetrics in the same institution before him. Graham Lusk remained in this post for the next 11 years. In 1909 he was selected as the successor to Austin Flint in the physiology post at Cornell University Medical College. He continued at this institution for the next 21 years and retired from his duties there only a few weeks prior to his death.

Dr. Lusk was married to May Tiffany in 1899. From this union came three children; William T., Louise (Mrs. Collier Platt) and Louis T. Mrs. Lusk contributed much to his success

through her sympathetic understanding of his work. Together they entertained his staff each year in their home for a dinner which was followed by a theater party. She invited his medical students to their home for Sunday afternoon teas. On a number of occasions, their summer home at Syosset on Long Island was a center for the entertainment of scientific groups. On these occasions the beautiful collections of art glass on the adjoining Tiffany estate were made available for inspection.

Most of the early work of Lusk was concerned with the subject of phlorhizin diabetes, or phlorhizin glycosuria as he preferred to call it. He published two papers from the Yale laboratories which were concerned with the action of this drug on the dog and on the rabbit. He observed that when the drug was continuously applied for several days to the fasting animal, a fixed ratio of 3.65 to 1 was obtained. From this it was deduced that protein is converted to carbohydrate to the extent of 58%. The application of the phlorhizin technic for the demonstration of intermediary protein and carbohydrate metabolism was subsequently widely employed by Lusk and his pupils. His collaborators in this work included P. G. Stiles, Arthur R. Mandel, A. I. Ringer and F. Csonka. It was demonstrated that amino acids such as glycine and alanine are completely converted to glucose, while only three carbons of aspartic or glutamic acids follow such a pathway. Lactic acid, pyruvic acid, methyl glyoxal, glyceric acid and other similar intermediates were shown to be sugar-formers. Deuel and W. H. Chambers in 1924 employed the phlorhizin technic for demonstrating the complete conversion of glycerol to glucose. As late as 1927 a discussion of the mechanism of action of phlorhizin gycosuria appeared in the *Journal of Biological Chemistry* under the authorship of Deuel, H. E. C. Wilson and A. T. Milhorat.

When Lusk took the post at Cornell, it was agreed that a respiration calorimeter should be constructed for study of the energy metabolism of dogs and of small babies. It was decided to model it after the Atwater-Rosa-Benedict type which was

in operation in the laboratory of F. G. Benedict in Boston. Dr. H. B. Williams, who was already on the Cornell staff, designed the instrument and supervised its construction. Lusk and Williams, together with J. A. Riche, formed a team which was essential for the operation of the instrument.

Shortly after the calorimeter was in successful operation, Williams left to accept an appointment to the College of Physicians and Surgeons of Columbia University. The numerous collaborators in the Cornell laboratories in the succeeding years included J. R. Murlin, A. I. Ringer, Eugene DuBois, R. J. Anderson, H. V. Atkinson, David Rapport, H. J. Deuel, Jr., and W. H. Chambers, as well as a number of students who were at Cornell for less extended periods. The results of their investigations were published in a series of 42 papers entitled *Animal Calorimetry,* many of which appeared in the *Journal of Biological Chemistry* from volume 12 to volume 100.

Most of the calorimeter studies were concerned with specific dynamic action, although some had as their object investigations of other factors related to energy metabolism. One especially interesting paper was that published with R. J. Anderson in 1917 on *"The Interrelation between Diet and Body Condition and the Energy Production during Mechanical Work."* In this research it was shown that the caloric expenditure by dogs in running was no greater after a prolonged period of fasting when fat was almost the sole source of energy than it was for well-nourished animals which had previously received glucose. These well-controlled and decisive experiments were completely overlooked somewhat later when it was claimed that carbohydrate had an efficiency about 11% higher than fat for the accomplishment of mechanical work in man.

However, the bulk of the respiration studies were related to the subject of the specific dynamic action of foodstuffs. It was believed that a "plethora" hypothesis could best explain the increased heat production which follows the ingestion of carbohydrate and fat, while the effect of protein in stimulating the energy metabolism was believed to be caused by the

presence of certain amino acids, of which glycine and alanine are the most powerful. Glutamic acid, on the other hand, exerted no dynamic effect either in the normal or phlorhizinized dog, a fact that was confirmed in one of Lusk's last publications in 1930, in which he collaborated with W. H. Chambers. While no completely satisfactory hypothesis for explaining the cause of the specific dynamic action of protein could be formulated, Lusk was inclined to accept the explanation of Adams based on the thermodynamics of the reaction. According to this investigator, the change of glutamic acid to urea and glucose takes place spontaneously without the necessity of outside energy. On the other hand, the metabolism of alanine to urea and sugar requires the expenditure of extra energy. The necessity for such additional heat would then be the cause of the specific dynamic action associated with the latter amino acid.

Lusk was most insistent that basal metabolism, under normal conditions where the diet is constant, is absolutely uniform. In support of his contention, he cited the many practically identical results on basal metabolism obtained on the trained calorimeter dog, "Lady Astor," over a period of several years. Similar data were given for the basal metabolism of himself and Eugene DuBois over a somewhat longer interval. Lusk would not accept as reliable work in which irregularities in the levels of basal metabolism were reported from day to day. Such results were rather to be ascribed to faulty technic. Not only was basal metabolism believed to be especially constant, but the specific dynamic action resulting from the administration of a given amount of glycine or gelatin was shown to be reproducible within very narrow limits. In fact, Lusk stated in a lecture at the Mayo Foundation that "The heat produced by mixing a given quantity of water and sulfuric acid together in a test-tube is scarcely more exactly measurable than are these reactions of the living cells to amino-acids or polypeptides which reach them after meat or kindred substances are taken as food."

Although Lusk's experiments were highly regarded by his scientific colleagues, they did not go unchallenged by some of the less well-informed laity. The report was circulated in the literature of the antivivisectionists that the Department of Physiology at Cornell University Medical School had an instrument, the calorimeter, for the torture of dogs in which they were roasted alive! Whoever started the report certainly never observed the care used with the trained calorimeter dogs nor the kindnesses showered on them.

Although Lusk was the author of a wide variety of scientific papers, he is probably best known for his book on "*The Science of Nutrition.*" This was first published in 1906 and three succeeding revisions appeared, the last one in 1928. Unfortunately the book is now out of print and many of our younger biochemists are unable to see this volume, which will always be a classic in the field of respiratory and intermediary metabolism. The scientific descent of the Voit school from Lavoisier given on the frontispiece is of especial interest in making one realize the fundamental background on which the teachings of Lusk were based. The book remains unique in its field. The only volume which can be compared with it is DuBois' book on "*Basal Metabolism in Health and Disease.*" It is most fitting that DuBois should now occupy the chair in Physiology at Cornell held for so many years by Graham Lusk.

There are several topics on which Dr. Lusk took an especially firm stand. Probably the one on which he felt most deeply concerned the alleged transformation of fat to carbohydrate. While he agreed to the evidence that glycerol or fatty acids (as propionic) with an uneven number of carbon atoms could be converted to glucose, he was equally insistent that natural fats composed of even-chain fatty acids were not convertible to carbohydrate. He debated any reports intending to demonstrate such a change with great vigor and conviction. In fact he once wrote that the high values for D:N ratio reported by Hartogh and Schumm for fasting phlorhizinized dogs were "unobtainable in a good laboratory." Had the figures of these investigators been valid, the only source of the extra sugar

would have been fat. He attacks with equal acrimony the reports of Grafe and Wolf of a high D:N ratio in fasting diabetic patients. In refutation of this type of evidence, he cites an experience of Dr. DuBois with one of his diabetic patients. After the nurse had been absent for only one minute during the visit of a relative, it was discovered that two sandwiches had been secreted in the bed. On further examination two more were found hidden in the recesses of his armpits. Lusk observes that "There is no question that this is the variety of 'proof' that diabetics produce sugar from fat." His criticisms of other types of evidence advanced in favor of the fat → carbohydrate change are equally severe. In this category are the reports of low R.Q.'s (below 0.69) and the theory that epinephrin controls the conversion of fat to sugar.

Another controversy continued for a number of years between Dr. F. G. Benedict and Graham Lusk. In this case the differences were based not on the correctness of the data obtained but on the interpretation of such results. One of the most extended disputes concerned whether "surface area" or "active protoplasmic mass" is the better biometric index for basal metabolism. We are now convinced that standards based on their consideration are satisfactory. Another difference of opinion involved the metabolism in diabetes. Benedict and Joslin had maintained that their metabolism of such patients is 15 to 20% above normal. When such data were calculated on the basis of surface area, Lusk showed that the diabetics had a basal metabolic rate of only + 2, although the controls used by Benedict and Joslin had a B.M.R. value of — 9. The difference in opinion arose as to whether emaciated controls who had an abnormally low metabolic rate should be used for comparison or whether normal subjects should have been chosen.

Lusk was associated with the formation and early development of most of the physiological and biochemical groups now well established. Although he was not one of the founders of the American Physiological Society, he became a member of that group at its 5th annual meeting in Princeton in 1892. He

was one of the charter members of the American Society of Biological Chemists and served as its chairman in 1914. On January 17, 1903, Lusk was one of a group of 7 who met at his home to organize the Society for Experimental Biology and Medicine. This organization was familiarly known for a long period as the "Meltzerverein," after another of the group who helped to organize it. Two years later (April 1, 1905), another meeting was called at Lusk's home to consider the formation of a society of clinicians, the aim of which was to assist in their appreciation of fundamental scientific work. In this group were included such representative scientists as J. J. Abel, E. K. Dunham, James Ewing, Simon Flexner, Christian Herter, T. C. Janeway, F. S. Lee, P. A. Levene, S. J. Meltzer, E. L. Opie, W. H. Park and G. B. Wallace. As a result of this meeting a new society was formed for the diffusion of knowledge of the medical sciences through the medium of annual courses of public lectures. At the suggestion of Professor Lusk, this group was named the Harvey Society. For the first 10 years, Lusk took an especially active part in the development of the Society, acting either as president or as a member of the Council. In 1908, together with Dr. J. J. Abel, Dr. Lusk acted as one of the sponsors in the launching of the American Society of Pharmacology and Experimental Therapeutics.

The true greatness of Lusk lay in his merit not only as a scientist but as a man. He was an example for younger men to emulate for his scientific accomplishments, for the honesty of his convictions, for his generosity and for his kindness. His position in science is a matter of record. His strict code of honesty is illustrated by the following anecdote. He had been informed by one of his close friends on the stock market that Corn Products stock was an excellent buy and was slated for an especially bright future. Lusk refused to avail himself of the opportunity of purchasing such stock, for he felt that if he held it no one would accept as valid any work he subsequently did on glucose.

He was especially generous financially with his less fortunate associates. Almost single-handedly he raised enough money for the entertainment and travel of his European colleagues when they came to America for the International Physiological Congress in Boston in 1929. He entertained many of them in his home during their stay in New York.

Lusk was kind to everyone. The extent of his kindness was not always recognized. One such example came to my attention a few years after Dr. Lusk's death. Dr. M. Wierzuchowski had earlier worked for a year in the Cornell laboratories as a Rockefeller Fellow from Poland. He had come to the conclusion as a result of his studies in Lusk's laboratories that the D:N ratio of fasting phlorhizinized dogs is not a fixed one. When he showed his paper to the "Professor," Dr. Lusk did not forbid him to publish it or force him to alter it. The paper appears in the *Journal of Biological Chemistry* as being from the Cornell laboratories, but with a footnote to the effect that the interpretation is that of the author rather than of Professor Lusk. However, I learned the important part of the story almost 10 years later, in 1935, when I met Dr. Wierzuchowski at the International Physiological Congress in Russia. He said, "Deuel, we who worked with Dr. Lusk should stick together. Do you know what he did for me? In spite of what I did there, Lusk told Parnas that I was a good man and to see that I obtained a good berth in Poland. Professor Lusk was a really great man."

His unselfishness was shown by his philosophy of life. When one of his assistants had an excellent opportunity elsewhere, his reaction was that "What is best for the individual, is best for the School." Thus, he recommended that the individual accept the apopintment, even though the loss of his services created a somewhat difficult situation for him.

During his life Lusk received many honors. Yale University conferred a Doctor of Science degree on him, while he received the degree of Doctor of Laws from the University of Glasgow. He was a member of the National Academy of Sciences, a Fellow of the Royal Society of Edinburgh, and a foreign

member of the Royal Society of London. During World War I, he and Professor Russell Chittenden served as the American representatives on the Interallied Scientific Food Commission.

Probably the honor which meant the most to Lusk came on his 60th birthday. Unknown to him, a testimonial dinner was arranged at the old Waldorf Astoria on Fifth Avenue and 34th Street. Here his friends and associates from widely scattered parts of the United States gathered to pay tribute to him. They presented him with a volume containing a photograph and a letter from each admirer. The dinner was a hugh success which will never be forgotten by the 100 or more guests who attended. Professor Dayton Edwards, at that time a member of Lusk's department, and another close friend of many years, Professor George B. Wallace of the Bellevue Medical School, were primarily responsible for arranging this worthwhile gathering.

One can only conclude that to have known Lusk was a privilege and a pleasure. One could not fail to grow in stature, both scientifically and socially, as the result of association with Graham Lusk.

HARRY J. DEUEL, JR.

EFFECTS OF DIETARY COMPOSITION ON TOOTH DECAY IN THE ALBINO RAT

JAMES H. SHAW

Harvard School of Dental Medicine and Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

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Sognnaes ('48) observed a high susceptibility to tooth decay among rats of the Long-Evans strain which were fed certain purified diets for periods of three to 5 months after weaning, provided that the mothers of these rats had been maintained on the purified diets throughout gestation and lactation. On the other hand, when rats of the same strain were born to females fed a diet of natural foodstuffs throughout the reproductive cycle, the teeth of the offspring were relatively immune to tooth decay even when the purified diet was fed for long periods after weaning. The former observation has made it possible to establish breeding colonies of the common laboratory rat which will yield offspring of sufficiently high caries susceptibility as to be suitable for standardized experimental manipulation. When the offspring of rat colonies maintained on natural diets are fed purified rations after weaning, their caries resistance is sufficiently high to prohibit their use in routine dental caries studies.

A preliminary series of investigations has been conducted to determine whether alterations in the experimental regimen which caused variations in the incidence and extent of carious lesions in the caries-susceptible cotton rat would produce similar, or different, results in the caries-susceptible laboratory rat. The following facts have been demonstrated experimentally with the cotton rat: (1) isocaloric substitution of fat and protein at the expense of sucrose resulted in sub-

stantial reductions in the incidence of tooth decay (Schweigert et al., '46; Shaw, '49a); (2) the use of either mineralized whole fresh or whole evaporated milk as the sole nutrient inhibited tooth decay (Schweigert et al., '46; Anderson et al., '47; Shaw, '49c); (3) the addition of 10% sucrose by weight to mineralized whole fresh or whole evaporated milk resulted in only slight increases in the dental caries experience (Anderson et al., '47; Shaw, '49c); (4) restriction in caloric consumption resulted in decreased incidence of tooth decay (Shaw, '48); and (5) the type of caging arrangement influenced the extent of tooth decay (Shaw, '49b). The purpose of the present paper is to report the results of similar experimental studies in which caries-susceptible common laboratory rats were used as the subjects.

EXPERIMENTAL

The common laboratory rats used in this series of investigations had been born to females of the Sprague-Dawley strain which were maintained throughout their reproductive lives on caries-producing ration 100 (Shaw, '49a). When this diet is fed to female rats of the Long-Evans strain throughout pregnancy and lactation, their offspring have been shown to have a high susceptibility to tooth decay (Sognnaes, '48). Similarly, the molar teeth of rats from earlier litters born to these females of the Sprague-Dawley strain developed a relatively high number of carious lesions when the offspring were maintained on ration 100 for suitable periods after weaning.

The compositions of the various purified diets used in this series of investigations are itemized in table 1. Unless otherwise stated, the rations were offered ad libitum for the duration of the experiment.

The first experiment was designed to test the effect of isocaloric increases of fat at the expense of sucrose, and was continued for a period of 14 weeks after weaning. The first group of 11 rats was maintained on ration 100, which contained 4.8% fat and 64.4% sucrose. The second group of 11 rats was fed diet 130, which contained 13.8% fat and 52.1%

sucrose. The third group of 10 rats was fed diet 150, containing 25.0% fat and 36.9% sucrose.

The second experiment was conducted for a period of 18 weeks to test the effect of a further decrease in sucrose content accomplished by an increase in the casein level. The control group of 13 rats was fed ration 100 while the experimental group of 7 rats was offered ration 550, which had the same fat content as ration 150 but an increase in protein level from 28.6% to 47.6%.

TABLE 1

Composition of the purified diets

CONSTITUENTS	RATION			
	100	130	150	550
	gm	gm	gm	gm
Sucrose	67	49	31	15
Casein [1] [5]	24	24	24	40
Corn oil [2]	5	5	5	5
Lard	..	8	16	16
Salt mixture [3]	4	4	4	4
1:20 liver concentrate	2	2	2	2
Whole liver substance	2	2	2	2
Fat content (%)	4.8	13.8	25.0	25.0
Carbohydrate content (%)	64.4	52.1	36.9	17.9
Protein content (%)	23.1	25.5	28.6	47.6

[1] "De-vitaminized" casein, Sheffield Farms, New York. Each 24 gm had had incorporated in them 350 μg of thiamine hydrochloride, 350 μg of riboflavin, 350 μg of pyridoxine hydrochloride, 2.0 mg of calcium pantothenate, 2.5 mg of nicotinic acid, 100 gm of choline chloride, 100 mg of inositol, and 30 mg of para-aminobenzoic acid.

[2] Mazola. Each 5 gm had had incorporated in them 1.1 mg of beta-carotene, 300 I.U. of irradiated ergosterol, 0.6 mg of 2-methyl-1,4-naphthoquinone, and 5.0 mg of alpha-tocopherol.

[5] J. Dent. Res., *26:* 47, 1947.

The third experiment provided a test with white rats on the effect of mineralized whole milk as the whole diet or as part of the diet on the incidence and extent of tooth decay. Four groups of 8 rats each were maintained for 14 weeks on the following dietary regimens, respectively: ration 100;

mineralized whole milk; mineralized whole milk plus 10% sucrose by weight; one-third of the caloric intake supplied by mineralized whole milk and two-thirds by ration 100. The mineralized whole milk was prepared daily from fresh pasteurized, homogenized milk. To each 100 ml of milk were added 0.5 gm of 1:20 liver concentrate powder and sufficient inorganic salts to supply 1.0 mg of ferrous iron, 0.1 mg of copper, and 0.1 mg of manganese. This mixture was stirred in a Waring Blendor until it was homogeneous. The diet of mineralized whole milk plus 10% sucrose was prepared by adding 10 gm of sucrose to 100 ml of the previously described mineralized whole milk mixture and blending thoroughly. Sufficient of the fluid mixtures was measured out to supply just the day's requirement for each rat. The latter procedure was helpful in that the distribution of the nutrients consumed by the rats was more nearly predictable than when surplus quantities were fed. No drinking water was supplied to the rats in groups 2 and 3. Drinking water was supplied to the rats in group 4 after the milk supplement was consumed.

The 4th experiment provided an experimental measure of the effect of mineralized evaporated milk as the whole diet or as part of the diet on the incidence and extent of tooth decay in white rats. Three groups of 4, 5 and 5 rats were maintained on ration 100, on mineralized evaporated milk and on evaporated milk plus 10% sucrose, respectively, for a period of 18 weeks after weaning. No drinking water was supplied to the rats in groups 2 and 3. The mineralized evaporated milk was prepared by dilution of the milk as drained from the can with an equal volume of tap water and then supplementing and blending in the same fashion as in experiment 4.

In the 5th experiment, the effect of caloric restriction on the development of carious lesions in the white rat was tested. Three groups of 8 weanling rats each were maintained for 14 weeks on the following caloric allocations: The rats in the first group were provided ration 100 ad libitum in weighed

amounts. Each rat in the second group was provided daily with enough of ration 100 to supply 60% of the calories consumed by a litter mate of the same sex in the first group. Each rat in the third group was supplied with 40% of the calories consumed by a suitable control rat.

In the 6th experiment, three groups of rats were maintained under different types of caging arrangements for 14 weeks to determine if these variations altered the incidence of tooth decay. The 5 rats in the first group were maintained in individual cages with floors of coarse wire mesh. The 6 rats in the second group were maintained in two cages which had frequently changed, soft wood shavings as the bedding material on a sheet metal floor. The 6 rats in the third group were housed in two cages with floors of coarse wire mesh.

In all experiments, weanling litter mates were divided as equally as possible among the various groups. Water was supplied freely to all animals except those fed milk diets as the sole source of nutrients. In all experiments except the 6th, the rats were housed in individual cages with screen bottoms.

At the end of the designated experimental periods the rats were killed by ether and the heads fixed in 95% ethanol. After 48 hours the skin and major muscle masses were cut off and the skulls prepared for evaluation of the number and size of carious lesions by the procedure developed for the cotton rat (Shaw et al., '44).

RESULTS

The average number of carious molars, the average number of carious lesions and the average extent of carious lesions for the various control and experimental groups of rats are tabulated in table 2. In addition, the standard error of each mean and the critical ratio of the difference between means to the standard error of the difference between means are recorded.

Throughout the first 5 experiments there was a striking similarity between the data obtained in these investigations

TABLE 2

Caries incidence of albino rats fed various purified diets, or milk, or evaporated milk

RATION	NO. OF WEEKS	NO. OF RATS	NO. OF CARIOUS MOLARS			NO. OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
			AVG.	S.E.M.[1]	C.R.[2]	AVG.	S.E.M.[1]	C.R.[2]	AVG.	S.E.M.[1]	C.R.[2]
100	14	11	6.6	0.6	3.1	8.2	0.9	3.0	16.6 +	1.3 +	4.0
130	14	11	3.5	0.8	5.1	4.3	1.0	4.7	7.7 +	1.7 +	7.4
150	14	10	2.6	0.5		3.0	0.6		4.8 +	1.3 +	
100	18	13	8.8	0.3	17.6	15.4	1.1	12.3	38.6 +	5.0 +	7.4
550	18	7	1.3	0.3		1.3	0.3		1.4 +	0.3 +	
100	14	8	4.5	0.4	9.8	4.8	0.4	10.7	8.3 +	1.3 +	6.0
Mineralized milk	14	8	0.4	0.1	10.0	0.4	0.1	10.5	0.5 +	0.1 +	5.9
Mineralized milk + 10% sucrose	14	8	0.5	0.1	1.9	0.5	0.1	1.9	0.6 +	0.1 +	1.2
Mineralized milk + 100	14	8	3.3	0.5	5.6	3.6	0.5	6.2	6.0 +	1.3 +	4.2
100	18	4	4.5	0.6	6.8	5.0	1.0	4.6	8.5 +	1.7 +	4.4
Mineralized evaporated milk	18	5	0.4	0.1	6.5	0.4	0.1	4.4	0.6 +	0.5 +	4.2
Mineralized evaporated milk + 10% sucrose	18	5	0.6	0.1	1.4	0.6	0.1	1.4	1.0 +	0.6 +	0.5
100	14	8	4.9	1.2		6.3	1.7		15.4 +	5.0 +	

100	14	8	4.9	1.2	2.4	6.3	1.7	2.5	15.4 +	5.0 +	2.4
100-intake restricted to 60%	14	8	1.5	0.7		1.6	0.8		2.8 +	1.5 +	
100-intake restricted to 40%	14	8	0.3	0.1	4.7 / 1.6	0.3	0.1	3.5 / 1.6	0.3 +	0.1 +	3.0 / 1.7
100 (rats in individual screen bottom cage)	14	5	6.4	0.9	1.3	7.8	1.2	0.2	20.2 +	3.2 +	1.4
100 (3 rats to cage, shavings as bedding)	14	6	5.0	0.6	0.8	7.5	1.4	0.2	13.8 +	3.5 +	0.4
100 (3 rats to cage with screen floor)	14	6	5.3	0.9	0.3	7.5	1.6	0	17.9 +	4.2 +	0.7

[1] Standard error of mean.

[2] Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant. The criss-cross lines in the above table connect sets of data which are being compared for the critical ratio test of significance.

with caries-susceptible white rats and those resulting from previous studies under similar conditions with caries-susceptible cotton rats.

As judged by the average number of carious molars, the average number of carious lesions and the average extent of the carious lesions, the administration of rations 130 and 150 with increased fat content and decreased sucrose content to albino rats throughout the period of investigation resulted in significantly lower average rates of tooth decay than were observed among the control litter mates fed ration 100 for the same period. Ration 130, with the lower fat and higher carbohydrate content, permitted the development of a higher degree of tooth decay than did ration 150. All three groups grew at approximately the same rates.

In the second experiment, the further reduction of sucrose by partial replacement with additional casein in a diet with a high fat content (ration 550) resulted in an almost complete prevention of tooth decay in this strain of rats. The difference between the control and experimental groups had the highest statistical significance of all differences resulting from the procedures tested in this series of studies. The animals receiving diet 550 grew slightly more rapidly and attained somewhat greater final weights than the controls fed ration 100.

The feeding of either mineralized fresh milk or mineralized evaporated milk as the sole source of nutrients in the third and 4th experiments resulted in an almost complete prevention of tooth decay in the common laboratory rat. The addition of 10% sucrose by weight to either mineralized fresh milk or evaporated milk did not appreciably increase the incidence of tooth decay. In both experiments the difference between the average incidence of tooth decay in the control groups, as judged by the three criteria mentioned above, was significantly higher than the average incidences of tooth decay in those rats fed the milk diets or the milk diets supplemented with 10% sucrose by weight. The rats on the milk diets grew considerably more slowly than those fed ration 100. The

addition of 10% sucrose resulted in an appreciably more rapid rate of growth. The group of rats in experiment 3 which were fed ration 100 and mineralized milk had a slightly, but not significantly, lower average incidence of tooth decay than the control animals. However, the average incidence of tooth decay in the rats fed ration 100 and mineralized milk was significantly higher than in the rats fed either mineralized milk or mineralized milk plus 10% sucrose. The average rate of growth for the animals fed ration 100 and mineralized milk was slightly slower than the rate for those fed ration 100 but was appreciably more rapid than for those fed the milk diets.

A significantly low average incidence of tooth decay was observed in the group of rats whose caloric intake had been restricted throughout the experimental period to 40% of the food consumption of the control rats. The rats whose caloric intake was restricted to 60% had an appreciably, but not statistically significantly lower dental caries experience than their control litter mates. The average rates of growth in the calorically restricted animals were necessarily very slow and they had not attained adult stature at the time they were sacrificed.

In the 6th experiment, the three groups of rats which were housed under slightly different caging arrangements had very similar incidences of tooth decay. Unlike the cotton rat, the type of caging did not appear to alter the tendency of caries-susceptible rats to develop carious lesions. The average rates of growth of these three groups of rats were almost identical.

DISCUSSION

This series of investigations indicates the similarity with which dietary manipulations affect the initiation and development of carious lesions in two caries-susceptible species of rodents — the cotton rat and the common laboratory rat. This similarity obtained despite the fact that there is a considerable difference between the general appearance and form of the molar teeth in the two species, especially in regard to the width and depth of the sulci in the molars. The demon-

stration that the initiation and development of carious lesions in these species are influenced similarly by the range of dietary variations reported above is of considerable importance in the planning and interpretation of experiments in which these species are used as the subjects. Since the crowns of the molar teeth of these rats were largely formed at the beginning of these experiments, it should be emphasized that the dietary effects studied in this series of investigations must have been brought about largely through post-developmental pathways.

The observation that caging arrangements did not influence the initiation and development of carious lesions in the white rat, contrary to a previous finding with the cotton rat, may have resulted from the difference in width and depth of the sulci of the molar teeth in the two species. Carious lesions in both the white rat and the cotton rat originate in the enamel at the base or on the sides of the sulci. Cotton rats maintained in cages with metal floors covered by wood shavings accumulate firm, densely compacted masses of wood fibres in the narrow, deep sulci of their molars. Under these circumstances, carious lesions develop infrequently and at a relatively slow rate. In contrast, the characteristic grooves of the molars of the white rat are considerably wider and more shallow than those of the cotton rat. Consequently, there is no appreciable accumulation of fibers in the sulci of the molars in the albino rat, and no demonstrable reduction in dental caries experience as a result of caging.

SUMMARY

Weanling caries-susceptible albino rats were used as the experimental subjects in a series of investigations to determine whether the initiation and development of carious lesions would be influenced by dietary procedures already demonstrated to alter the incidence of tooth decay in caries-susceptible cotton rats.

The isocaloric substitution of fat and protein for part of the sucrose in the purified ration resulted in substantial re-

ductions in the incidence of tooth decay in comparison to that in the control rats. Administration of either mineralized fresh or evaporated milk as the sole source of nutrients resulted in extremely low degrees of dental decay. The addition of 10% of sucrose by weight to either mineralized fresh or evaporated milk did not result in any appreciable increase in tooth decay above the average for the animals on the comparable milk diets alone. Caloric restriction resulted in appreciably less tooth decay than in the control rats which were allowed to eat ad libitum. In each of the above results, the influence of dietary variation on the initiation and development of carious lesions in the caries-susceptible white rat is extremely comparable to the effect obtained with the same regimens in cotton rats.

In contrast, the type of caging arrangement employed throughout the experimental period was not found to influence tooth decay in the white rat, unlike its strong influence in the cotton rat.

ACKNOWLEDGMENTS

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EFFECT OF ENVIRONMENT ON GROWTH AND FEED AND WATER CONSUMPTION OF CHICKENS

III. THE EFFECT OF TEMPERATURE OF ENVIRONMENT DURING THE PERIOD FROM 18 TO 32 DAYS OF AGE

H. G. BAROTT AND EMMA M. PRINGLE

Animal Husbandry Division, Bureau of Animal Industry, U. S. Department of Agriculture, Agricultural Research Center, Beltsville, Maryland[1]

TWO FIGURES

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INTRODUCTION

The first two papers of this series (Barott and Pringle, '47, '49) showed the effect of temperature on the growth of chickens and the efficiency of feed utilization from time of hatch to 18 days of age. The present paper covers the succeeding two weeks, from the 18th to the 32nd day of age.

PROCEDURE

This work was conducted in the three small houses with environmental control which were fully described in the first paper. The same equipment and feed formula, and chicks from the same breeding stock, were used.

During the latter part of 1948 a 4th experimental house identical with the other three was constructed. It was used for the first time in an experiment performed in December, 1948. This house allows for the simultaneous conducting of 4 experiments, thus expediting the work by one-third.

[1] Acknowledgment is made of the assistance of James Jackson, who aided in the general routine of conducting these experiments.

The undigested feed in the system was obtained as formerly and the amount applied as a correction to the figure showing apparent growth. This correction increased lineally from 11.30 gm on the 18th day to 17.60 gm on the 32nd day.

The temperature for the first 18 days after hatch was that reported in the first two papers — 94° to 95°F. at the start, dropping uniformly to 80°F. on the 18th day. The temperature on the 32nd day was varied from 76° to 55°F. Thirty-one experiments in all were performed and 30 chicks were used in each.

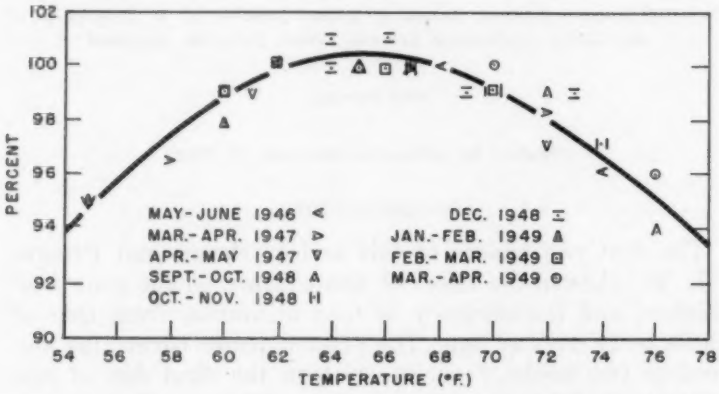

Fig. 1 Effect of temperature on growth of chicks between the ages of 18 and 32 days expressed as per cent of growth at optimum temperature.

RESULTS AND DISCUSSION

Figure 1 shows the proportional growth in each experiment. As was stated previously, three or 4 experiments were conducted simultaneously. In each case the growth obtained in the house with an end temperature nearest 65°F. was taken as 100%, and the values obtained from the other experiments performed at the same time were computed as a percentage of this value and so plotted.

A curve drawn through the plotted points for these 31 experiments shows maximum growth at about 66°F. This in-

dicates that the best growth for chickens between the 18th and 32nd days of age is obtained when the temperature is 80°F. on the 18th day and drops uniformly each day thereafter so that on the 32nd day it is 66°F. As the temperature varied in either direction from this figure the growth was less and it became poorer and poorer the greater the variation.

With chickens of this age a variation in temperature is not nearly as critical as for younger chicks. For chicks between zero and 18 days of age, a variation in the temperature on the 18th day from 70° to 80°F. made a difference in growth of 35%, whereas with chickens between 18 and 32 days of age a variation from 55° to 65°F. made a difference in growth of only 6%. Chickens at 32 days of age are becoming more fully feathered and are better able to adapt themselves to temperature changes. A variation in temperature for still older chickens would be of even less importance; consequently, it is the intention of the present investigators to discontinue the research on temperature at this point, as the critical stage relative to temperature control has been covered.

The 10 experiments that were conducted with an end temperature of from 64° to 68°F. were used to compute the values from which the curves in figure 2 were plotted.

Curve 1 shows the increase in weight in grams per chicken per day. This curve appears to be nearly linear after the 12th day, each day's gain being about 0.6 gm greater than that of the previous day.

Curves 2 and 3 show, respectively, the amount of feed eaten and water drunk in grams per chicken per day. These curves increase rather sharply between the 18th and the 26th days. The slope after the 26th day is about twice that before the 18th day.

As the feed eaten increases more rapidly than the growth, the efficiency after the 18th day would necessarily decrease. This is clearly shown in curve 4. Here the efficiency of feed utilization increases until the 18th day and then decreases until the 32nd day. We know that the efficiency of feed utilization for growth (as we have computed it) must become

zero when growth ceases. A pullet reaches maturity at about 22 to 24 weeks of age and growth after this time will be small, although the bird may attain considerable weight by fat deposition. The birds at the termination of our study were nearly 5 weeks of age or had reached about 25% of

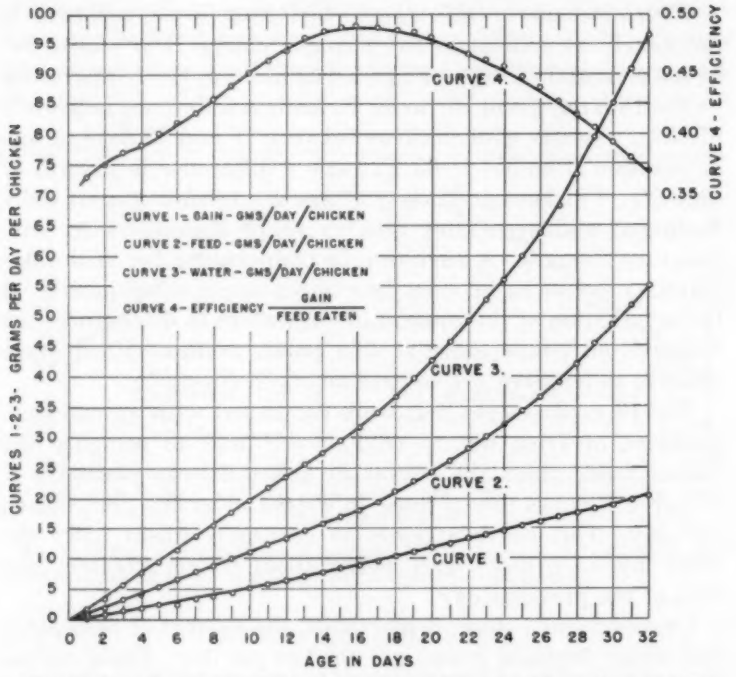
CURVES 1-2-3- GRAMS PER DAY PER CHICKEN
CURVE 4 - EFFICIENCY
AGE IN DAYS
CURVE 1= GAIN - GMS/DAY/CHICKEN
CURVE 2- FEED - GMS/DAY/CHICKEN
CURVE 3- WATER - GMS/DAY/CHICKEN
CURVE 4- EFFICIENCY GAIN / FEED EATEN
CURVE 4.
CURVE 3.
CURVE 2.
CURVE 1.

Fig. 2 Growth and feed and water consumption of chickens from hatch to 32 days of age.

maturity. As the efficiency curve decreases during the last two weeks of this 5-week period, it is logical to assume that the efficiency will continue to decrease until maturity is reached.

The amount of water drunk seems to be in almost direct proportion to the amount of feed eaten, and equals approxi-

mately 1.75 gm for each gram of feed consumed. The values obtained for water consumption are undoubtedly somewhat high. Although a correction was made for evaporation from the water trough, a chicken will waste some water while drinking by flipping its head and casting drops away. This cannot be collected, as in the case of the feed, but evaporates into the air. Also a chicken occasionally steps into the drinking trough and carries away some water by so doing. However, these errors are small compared with the amount of water drunk after the first few days; nevertheless, they tend to make the amount of water as determined somewhat high.

SUMMARY

Chickens were housed in a controlled environment without a hover for the first 32 days after hatch to determine the optimum temperature for growth and efficiency of feed utilization from the 18th to the 32nd day. Thirty chickens were used for each experiment and a total of 31 experiments was performed. Equipment, feed formula and routine of procedure were the same as for the work reported in previous papers by the authors ('47, '49).

The temperature during the first 18 days was that found to be optimum as reported in the first two papers: i.e., it was 94° to 95°F. at the start and then dropped uniformly to 80°F. on the 18th day. For the period from the 18th to the 32nd day the temperature was varied with different lots of chickens from 80°F. on the 18th day to from 76° to 55°F. on the 32nd day.

Maximum growth from the 18th to the 32nd day was found when the temperature dropped uniformly from 80°F. on the 18th day to 66°F. on the 32nd day. The growth became less as the temperature varied either way from that noted for maximum growth, and the greater the variation the greater the decrease.

The efficiency of feed utilization dropped from a value of 0.49 on the 18th day to 0.38 on the 32nd day.

The water drunk was approximately 1.75 gm for every gram of feed eaten.

It was decided to discontinue research on temperature at this point, as the critical phase relative to temperature has been covered.

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GERM-FREE CHICKEN NUTRITION

I. GROSS DEVELOPMENT AND VITAMIN UTILIZATION STUDIES EMPLOYING WHITE LEGHORN CHICKS

J. A. REYNIERS, P. C. TREXLER, R. F. ERVIN, M. WAGNER, H. A. GORDON AND T. D. LUCKEY

Germ-Free Life Division of LOBUND, University of Notre Dame, Notre Dame, Indiana

AND

R. A. BROWN, G. J. MANNERING AND C. J. CAMPBELL

Research Laboratories, Parke, Davis and Company, Detroit, Michigan

ONE FIGURE

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The contribution of intestinal microorganisms to the nutrition of the host presents an important problem in the interpretation of nutritional data. Interest has centered on the intestinal inhabitants as producers of vitamins. Indirect methods employed to control the microbial population include surgical procedures and the feeding of sulfanamides, antibiotics and special diets. While these studies have yielded valuable information, they not only fail completely to eliminate or control the variable being studied but they introduce factors which may affect the response being observed in the host. The direct approach to problems dealing with interrelationships between intestinal microorganisms and host is the germ-free technique.

Several investigators have attempted to rear germ-free chicks on practical diets (see review of Reyniers et al., '49a), but techniques had not been developed sufficiently to permit a broad experimental program. The pioneering efforts of Nuttal and Thierfelder (1897) to rear germ-free chicks were unsuccessful. Schottelius ('13) maintained life in several

germ-free chicks for short periods of time. Cohendy ('12) and Balzam ('37) reported growth rates of germ-free chicks to be comparable to those of control chicks. Balzam showed that there was no essential difference in the course of deficiency between germ-free and control chicks fed a diet low in B vitamins. More recently, Reyniers et al. ('49a, b) have reported that germ-free chicks grew well, matured and reproduced when fed diets of the so-called "synthetic" type fortified with yeast extract and liver.

The purpose of the following experiments was to determine whether germ-free chicks require unidentified vitamins which might be supplied either from dietary sources or by intestinal microorganisms. Special consideration was given to changes in the composition of diets during sterilization and to a comparison of the quantities of vitamins found in the livers and cecal contents of germ-free and control chicks.

EXPERIMENTAL

The method used for rearing germ-free chicks was that of Reyniers et al. ('49a). In brief, clean, White Leghorn eggs with 18- or 20-day-old viable embryos were taken from the incubator, scrubbed lightly with a detergent, rinsed, submerged in a 1% $HgCl_2$ bath for 8 minutes at 38°C. and passed through a $HgCl_2$ trap into a steam sterilized germ-free unit for hatching and rearing. Egg shells and unhatched eggs were removed, and food, water, and culture media were taken in through the sterile lock with steam sterilization. Sterile air was obtained by filtration through glass wool.

Control chicks were hatched in a standard incubator and reared in a conventional manner. All chicks were fed and watered ad libitum within 48 hours after hatching.

At weekly intervals samples of feces, food and water were collected in sterile test tubes inside the cage. Moist cotton-tipped applicators were used to swab the oral, anal, skin, feather, inner cage and glove surfaces. Multiple samples were taken from each source so that cultivation under a variety of enviromental conditions and direct microscopic ex-

amination could be accomplished. The samples were passed out of the cage through the sterile lock. Direct microscopic examinations were made of wet mounts and Gram-stained smears. Cultivation was done in deep tubes containing fluid thioglycollate medium and petri plates of 5% sterile, defibrinated horse blood in a brain-heart infusion agar base. Multiple tubes and plates were inoculated from each sample source so that each could be incubated under aerobic and anaerobic conditions and at temperatures of 25°, 37.5°, and 50°C. All cultures were incubated for two weeks before final observations were recorded. If evidence of contamination was observed, the results were not used in the compilation of data.

The analytical methods used were as follows: The material to be assayed was submitted to a general enzymatic hydrolysis (Cheldelin et al., '42), neutralized and filtered. Thiamine and choline were determined by chemical methods (U.S. Pharmacopoeia, '47). Nicotinamide and pantothenic acid were determined microbiologically (*L. arabinosis*) with slight modifications in the method described by the Association of Vitamin Chemists ('47). Biotin, riboflavin and folic acid were determined microbiologically using a modification of the *L. casei* method of Luckey et al. ('44). Pyridoxine was measured by means of the response of *S. carlsbergensis* (Atkin et al., '43). Vitamin A was determined using a semi-micro adaptation of the Carr-Price reaction according to the method of Dann and Evelyn ('38). Vitamin E was assayed by the method of Rawlings ('44). Vitamin K was estimated with a chick bioassay method which was essentially that of Thayer et al. ('39). Lysine was determined microbiologically as described by Henderson and Snell ('48). Tryptophan was assayed by the procedure of Horn and Jones ('45).

The composition of the diets is given in table 1. The diets were layered one-quarter to one-half inch thick in gauze containers to allow proper steam penetration, placed in the sterile lock and sterilized with saturated steam at 17 pounds per square inch gauge pressure (123°C.) for 30 minutes.

This was preceded and followed by the maintenance of a vacuum of 26 inches of mercury for 10 and 30 minutes, respectively. Oil supplements and instruments were presterilized in an oven at 180°C. for one hour before being

TABLE 1

Composition of diets (gm/kg)

CONSTITUENT	56227	4415	L-142	L-165
Salts	50 [1]	50 [1]	47 [1]	60 [2]
$MnSO_4 \cdot 4H_2O$	1	1	1	..
Cellu flour	..	30	..	..
Starch, corn	549	520	518	450
Lactose	0.9	0.9	0.9	40
Lard	40	40	37	..
Oil, corn	3	..	..	60
Casein, purified	250	250	235	250
Gelatin	100	100	94	100
l-Cystine	3	3	3	2
Vitamin A (I.U.)	50,000	45,700	50,000	4,000
Thiamine HCl	0.016	0.008	0.016	0.06
Riboflavin	0.016	0.016	0.016	0.03
Nicotinamide	0.05	0.01	0.05	0.05
Choline HCl	2.0	2.0	2.0	2.0
Na pantothenate	0.044	0.02	0.044	0.2 [3]
Pyridoxine HCl	0.012	0.012	0.012	0.2
Biotin	0.0004	0.0004	0.0004	0.0004
Folic acid	0.005	0.005 [4]	0.005	0.01
Inositol	1.0	1.0	1.0	1.0
p-Aminobenzoic acid	0.1	0.3	0.1	..
Bacterial residue (BY-21)	..	..	20	..
Yeast extract, Difco	..	..	20	20
Liver, whole powder	..	..	20	20
Ascorbic acid (powder)	..	..	..	2
Vitamin D (A.O.A.C. units)	5,000 [5]	4,750 [5]	5,000 [5]	500 [6]
Vitamin E	2 [7]	0.17 [7]	2 [7]	0.25
Vitamin K	0.05	0.034	0.05	0.05

[1] Jones and Foster salt mix no. 12 (Jones and Foster, '42).

[2] The composition of LOBUND salts L-11 in grams per kilogram diet is: $CaCO_3$, 18.0; $CaHPO_4$ 3.3; K_2HPO_4, 13.5; Na_2HPO_4, 12.0; NaCl, 3.0; KI, 0.045; $MgSO_4 \cdot 7H_2O$, 4.5; $MnSO_4 \cdot 4H_2O$, 0.75; $Fe(C_6H_5O_7)_2$, 4.5; $CuSO_4 \cdot 5H_2O$, 0.23; $CoCl_2 \cdot 6H_2O$, 0.03; $ZnSO_4 \cdot 7H_2O$, 0.06; $Na_2B_4O_7 \cdot 10H_2O$, 0.03; and $AlK(SO_4)_2 \cdot 10H_2O$, 0.045.

[3] Ca salt.

[4] Norite eluate $\cong$ 500 μg per 100 gm diet. (Made from Armour's dried 70% ethanol-insoluble, hog liver residue; dilute with 15 parts water, adsorb at pH 3.0 with norite, filter, elute with ammoniacal 50% ethanol and concentrate the eluate *in vacuo*.)

[5] Fish liver oil.

[6] Vitamin D_3, crystalline.

[7] Thirty-five per cent mixed tocopherols.

placed aseptically into the sterile lock. The fresh liver extract was filtered through a clarifying filter, then through a Seitz bacterial filter, and passed aseptically into the germ-free unit via a second Seitz filter.

Food, water, and other materials were taken into the germ-free units about once a week.

The designation and the rearing conditions of the chicks are:

Group A — Germ-free chicks fed sterilized, experimental diets in a germ-free unit.

Group B — Control chicks hatched from germicide-treated eggs, fed sterilized, experimental diets, reared in a commercial starter brooder.

Group C — Control chicks hatched from germicide-treated eggs, fed non-sterilized, experimental diets, and reared in a brooder as in group B.

Group D — Control chicks hatched from non-treated eggs, fed sterilized experimental diets, and reared in a brooder.

Group E — Control chicks hatched from non-treated eggs, fed a non-sterilized diet, and reared in a brooder.

RESULTS

Gross observations

Observations obtained from 4-week-old germ-free chicks and their controls are summarized in table 2. Experiments are considered individually because of seasonal and dietary changes occurring during the three years of experimentation.

In the first experiment the growth of the germ-free chicks compared well with that obtained in the control groups. A comparison of group B with group C indicates that sterilization did not appreciably harm the diet. Chicks from unsterilized eggs (groups D and E) and chicks from sterilized

TABLE 2

Summary of mortality, growth, feather development and perosis at 4 weeks

EXPT.	GP.[1]	DIET	NO. CHICKS		WEIGHT	FEATHER DEVELOPMENT[2]	PEROSIS[3]
			Start	End			
					gm		
1	A	4415[4]	5	3	263 (248–283)	Good	$2P_1$
	B	4415[4]	6	5	236 (142–314)	Fair	$2P_1$,$1P_s$
	C	4415[4]	5	3	220 (201–234)	Good	$1P_1$
	D	4415[4]	4	4	255 (186–298)	Good	$1P_1$
	E	4415[4]	3	3	199 (127–235)	Fair	None
	E	4245[4]	4	4	263 (234–300)	Good	None
2	A	7345[4]	4[5]	2	230 (222, 237)	Good	None
	B	7345[4]	5	4	259 (224–300)	Good	None
	E	4245[4]	4	4	257 (202–283)	Fair	None
3	A	7345[4]	3	3	231 (193–253)	Fair	$1P_2$,$1P_s$
	B	7345[4]	6[5]	6	284 (288–320)	Good	$3P_s$
	E	4245[4]	6	6	290 (268–350)	Good	None
4	A	L-142	4	1	298	Fair	None
	A	L-142 + liver[6]	5	5	264 (213–295)	Good	None
	B	L-142 + liver[6]	4	4	272 (217–324)	Fair	None
	E	4245[4]	4	4	251 (227–283)	Good	None
5	A	L-142	6	5	248 (208–298)	Fair	None
	AB	L-142	4	4	199 (187–213)	Fair	None
	B	L-142	4	4	215 (193–233)	Fair	$2P_s$
	E	4245[4]	8	6	226 (138–270)	Fair	None
6	A	L-165	2	2	258 (247, 270)	Excellent	None
	A	L-165 + liver[6]	3	3	287 (233–327)	Excellent	None
	B	L-165	6	6	239 (165–267)	Good	None
	E	4245[4]	6	4	184 (170–201)	Good	None
7	A	56227	3[5]	2	287 (282, 292)	Excellent	$1P_s$
	A	56227 + 50 μg B_{12}/day	3	3	318 (270–365)	Excellent	None
	B	56227	2	0	..	..	..
	E	4245[4]	6[5]	3	230 (219–250)	Fair	None

[1] Explanation of groups is given in text.

[2] Feather evaluation scale:

excellent = full smooth feathering over all the body;
good = feathering almost complete but not smooth;
fair = incomplete development;
poor = undeveloped and ragged; and
bad = very few feathers present.

[3] Perosis evaluation: number preceding P is the number of chicks involved, the number behind P evaluates the extent: 1 = swelling, 2 = twisting or bending of metatarsus, 3 = slipping of tendon, 4 = inability to use lower part of leg, and s = slight swelling.

[4] Diet 4245 was a commercial broiler ration obtained from the Larrows Milling Co. and fortified with 3% whole liver powder and 3% dried brewers' yeast. It was not autoclaved. The other diets were similar to diet 4415, with the following differences: Diet 7345 contained 0.005 gm of synthetic folic acid per kilogram in place of the equivalent folic acid in norite eluate. The vitamins of diet 4415 were given by oral supplement weekly. They were mixed in the other diets before autoclaving.

[5] One runt discarded from this group.

[6] Oral supplement of extract from fresh, enzyme digested (1% trypsin, pH 8, Na_2CO_3, for 24 hours at 37°) pork liver $\cong \frac{1}{2}$ gm liver twice weekly.

eggs receiving identical diets (groups B and C) developed comparably, showing that sterilization of the egg surface did not harm the embryo. Feathering was acceptable in all groups and mild perosis occurred in all groups except groups E. The development of perosis in germ-free chicks shows that this syndrome is not necessarily of bacterial origin — a finding in agreement with present day thinking.

The growth rate of the germ-free chicks in the second experiment was within the range of the control chicks, but the average was somewhat lower. Feather development was good and perosis did not occur in any of the groups.

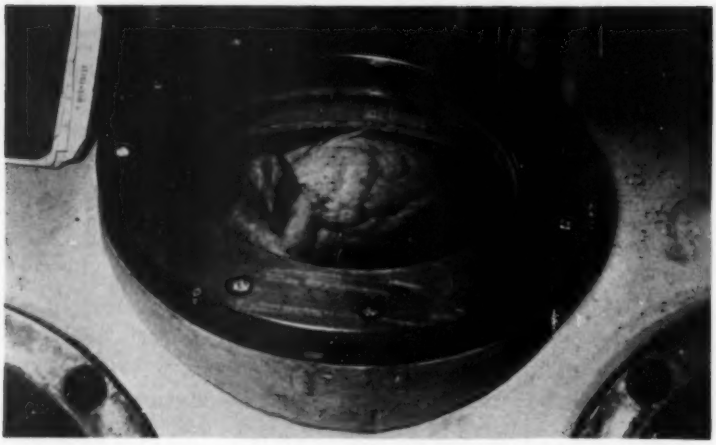
The results in the third experiment were similar to those of the second: The growth rate of the germ-free birds was slow but remained within the range of variation of the controls; feathering was fair and perosis was frequent.

In the 4th experiment germ-free chicks fed fresh, enzyme digested, Seitz-filtered liver extract grew no better than the survivor of the germ-free chicks fed the same diet without the supplement. All of the groups grew at about the same rate and none showed perosis.

The feathering in the 5th experiment was not as good as usual. The germ-free chicks grew at a slightly more rapid rate than the other groups. Chicks taken from the germ-free group when 48 hours old (group AB) did not grow as well as the chicks maintained germ-free.

In the 6th experiment, germ-free chicks given the fresh liver supplement by mouth grew 30 gm more than germ-free chicks supplemented with water only. The germ-free chicks again grew somewhat more rapidly than their controls. Feather development was excellent and no perosis occurred.

The control chicks in the 7th experiment did not develop well. All of the chicks in group B and two of the chicks in group E died during the first week of life. The growth of the germ-free birds was superior to that of the chicks in group E. Germ-free chicks which received extra thiamine (100 μg every other day by mouth) grew somewhat faster than the other germ-free chicks; however, the thiamine contents of



Pullet (Wgt. at 4 weeks — 365 gm)

Cockerel (Wgt. at 4 weeks — 292 gm)

Fig. 1 Germ-free chicks fed diet 56227.

the livers in both groups were about the same (table 6). The appearance of the germ-free chicks from this experiment is shown in figure 1.

A summary of the 8 experiments shows that the 16 germ-free chicks which received liver weighed an average of 265 gm and the 13 germ-free chicks receiving no liver grew to 267 gm at 4 weeks. Twenty-two of these 29 germ-free chicks may be compared directly with 33 control chicks which received identical diets. The average weight of the two groups at 4 weeks was 245 and 246 gm, respectively.

TABLE 3

Gross observations at 6 to 8½ weeks [1]

EXPT.	AGE	GROUP	DIET	SEX	NO. CHICKS	WEIGHT	FEATHER DEVELOPMENT	PEROSIS
	weeks					*gm*		
3	6	A	7345	♀	1	224	Fair	P_1
		A	7345	♂	2	487(481, 493)	Fair	P_3P_4
		B	7345	♀	2	482(461, 502)	Good	None
		B	7345	♂	4	471(295–572)	Good	$2P_4$
		E	4245	♀	3	457(419–480)	Good-excellent	None
		E	4245	♂	3	567(524–587)	Good	None
4	8½	A	L-142 + liver	♀	3	677(617–723)	Fair	None
		A	L-142 + liver	♂	1	834	Fair	None
		B	L-142 + liver	♀	1	593	Poor	None
		E	4245	♀	3	738(677–786)	Fair-poor	None

[1] See table 2 for explanation of symbols.

Two of the experiments were continued for 6 to 8½ weeks. The results (table 3) were quite similar to those previously described. One of the germ-free chicks did not grow well; otherwise, these chicks were very similar to the control birds.

Hematopoiesis

Observations on hematopoiesis were made at the termination of the experiments. No consistent differences were seen in the hemoglobin, hematocrit, or total red blood cell values

TABLE 4

Hematopoiesis in germ-free and control chicks

EXPT.	GRP.	AGE	NO. CHICKS	Hb	HEMATOCRIT	RED BLOOD CELLS
		days		*gm/100 cm³*	*%*	*10⁶/mm³*
1	A [1]	26	3	8.63 (8.2–9.0)	29 (27–30)	2.34 (2.09–2.44)
	B	26	5	8.98 (7.9–10.0)	31 (29–33)	2.82 (2.41–3.52)
	C	26	3	8.48 (7.7–8.9)	31 (27–33)	2.22 (2.03–2.38)
	D	26	4	9.00 (8.7–9.4)	30 (30–31)	2.78 (2.42–2.94)
	E	26	3	9.60 (9.0–10.2)	30 (26–34)	2.73 (2.13–3.67)
	E	26	4	8.56 (8.4–9.6)	31 (29–36)	2.94 (2.35–3.72)
2	A	28	2	8.74 (8.60–8.88)	29.5	3.16 (2.92–3.40)
	B	28	2	8.53 (8.60–8.46)	35.5 (33.0–40.0)	3.19 (2.73–3.80)
	E	28	9	8.67 (8.05–9.32)	32.9 (26.0–36.5)	2.76 (2.40–3.26)
3	A	27	2	8.78 (8.67–8.88)	30.5 (30.5–30.5)	2.98 (2.95–3.00)
	B	27	4	8.94 (8.60–9.39)	33.4 (32.5–34.5)	3.08 (2.76–3.51)
	D	27	4	8.64 (8.05–8.88)	32.5 (32.0–33.0)	2.49 (2.37–2.74)
	E	27	4	8.61 (7.58–9.17)	31.3 (28.0–34.0)	2.72 (2.41–2.92)
4	A	43	1	10.80	36.5	3.12
	B	43	3	7.98 (7.78–8.32)	27.0 (26.0–28.0)	2.49 (2.18–2.81)
	E	43	3	8.62 (8.18–9.23)	30.2 (29.0–31.5)	3.01 (2.83–3.27)
5	A	41	2	8.93 (8.13–9.73)		3.07 (2.88–3.25)
	B	41	2	8.99 (8.91–9.06)		3.12 (2.86–3.37)
7	A	31	2	9.07 (8.91–9.22)		2.94 (2.93–2.94)
	A [2]	31	3	8.80 (8.63–8.91)		2.98 (2.62–3.18)
	B	31	4	9.15 (8.67–9.47)		3.41 (3.10–3.66)
	E	31	4	7.61 (6.76–8.09)		2.92 (2.70–3.40)
8	A	28	3	8.87 (8.13–9.34)		3.29 (2.92–3.12)
	A [3]	28	3	7.26 (6.57–7.87)		3.14 (2.59–3.80)
	E	28	3	10.01 (9.22–11.21)		3.37 (3.20–3.46)

[1] Germ-free group.
[2] Received fresh liver extract = 1 gm liver each week.
[3] Received extra thiamine by mouth.

(table 4). A summary of the white blood cell data from 4-week-old chicks is given in table 5. The results indicate that the total number of white cells was less in germ-free birds than in the control chicks. The high average in group C is probably without significance, since the average of the three chicks reported in this group was markedly affected by one high value. There is considerable overlapping of differential counts when group A is compared with other groups.

Utilization of vitamins

Limited studies were made of the vitamin contents of the livers of the germ-free and control (group B) chicks fed identical diets without yeast and liver supplements. The data (table 6) indicate that absorption and liver storage of vitamin A, folic acid, pantothenic acid and riboflavin were comparable in germ-free and control chicks. Germ-free chicks which were given 100 μg of thiamine every other day had no more liver thiamine than unsupplemented chicks. The ascorbic acid content of the adrenal glands of both groups was similar but the average value for the liver of germ-free chicks appeared to be somewhat low. This is of special interest, since suckling germ-free rats show improved growth and survival when fed vitamin C (Reyniers et al., '46) and laboratory chicks show improved development when vitamin C is included in synthetic-type diets (Briggs et al., '44).

The substantial quantities of B vitamins found in the ceca of germ-free chicks (table 6) show that either complete absorption of these water-soluble vitamins does not occur or that considerable quantities are excreted into the gastrointestinal tract. It is certainly a misconception to consider the vitamin content of the lower intestine to be wholly of bacterial origin. Rather, it appears that much, if not most, of the vitamins found in the cecum may represent either a lack of absorption or a re-excretion into the gastrointestinal tract. Microorganisms may use, synthesize, conjugate or de-

TABLE 5

White blood cell data for 4-week-old White Leghorn chicks

GROUP	NO. CHICKS	WHITE BLOOD CELLS	DIFFERENTIAL LEUKOCYTE COUNT				
			Lymphocytes	Monocytes	Eosinophils	Basophils	Heterophils
		1,000/mm³	%	%	%	%	%
A	14	16.3	67.4	3.4	0.6	4.0	24.6
		(7.4–23.4)	(53.7–83.0)	(0–10)	(0–2)	(2–9)	(12–41)
B	9	23.3	69.9	2.5	2.3	2.5	23.8
		(10.0–33.7)	(38.2–87.0)	(1–8)	(1–8)	(1–5)	(9–52)
C	3	42.5	76.0	1.7	3.7	1.7	17.0
		(20.5–85.8)	(73.1–78.8)	(1–2)	(2.6)	(1–2)	(14–21)
D	4	31.2	73.6	1.7	2.7	1.9	20.3
		(24.4–38.6)	(63.0–84.8)	(0–3.5)	(0.5–7)	(0–4.5)	(11–30)
E	11	25.2	76.5	3.9	1.6	2.8	20.0
		(11.1–31.6)	(62.0–92.0)	(0–9)	(0–5)	(1–7)	(6–69)

TABLE 6

Vitamin content of liver and cecum in 4-week-old chicks (expressed in μg/gm)

	LIVER (WET)				CECUM (WET)			
	Germ-free		Control		Germ-free		Control	
	No.	Ave.	No.	Ave.	No.	Ave.	No.	Ave.
Vitamin A	2	45 (42, 48)	2	27 (19, 35)				
Thiamine	2	4.64 (4.41, 4.86)	2	4.42 (4.11, 4.72)[1]				
Riboflavin	4	40 (25–50)	3	43 (26–56)	2	68 (44, 92)	2	48 (45, 51)
Ca pantothenate	3	83 (77–93)	3	65 (59–77)	3	12 (7–13)	3	46 (32–55)
Folic acid	5	2.1 (1.0–3.0)	5	1.9 (1.1–3.0)	3	3.1 (1.9–5.0)	3	6.8 (4.6–8.2)
Vitamin B_{12}					1	0.14	1	0.36
Vitamin C	12	172 (64–235)	5	254 (114–375)	7	881 (430–1375)[2]	3	943 (596–1202)[2]

[1] These were germ-free chicks fed the same diet but receiving oral supplements of 50 gm thiamine/day.

[2] Total ascorbic acid in adrenal tissues determined by method of Roe and Keuther ('43).

stroy vitamins in the intestinal canal, but it appears to be quite possible that the net effect may be very little different than if no intestinal microorganisms were present. Similar data have been reported for adult germ-free bantam chickens (Reyniers et al., '49c).

Stability of nutrients during steam sterilization

The vitamins in the experimental diets were added in excess because the amount of destruction during steam sterilization and subsequent storage at room temperature was unknown when the diets were first compounded. It was important to learn the extent of destruction of nutrients during these procedures. As would be expected (table 7), thiamine was the most heat-labile nutrient in the diet. However, no thiamine was lost when it was autoclaved in a weak acid solution (supplement 108A). Some riboflavin was lost during autoclaving but none was lost during storage. Less pantothenic acid was lost than was anticipated; about three-fourths remained after autoclaving and storage. When the analyses for nicotinamide and biotin were made after extraction of fat and fatty acids, the data were not appreciably different. The use of liberal quantities of vitamin E and evacuation of the air before steam treatment helped to prevent the oxidation of biotin, folic acid and vitamin A. Although analyses for vitamin D were not run, it was evident from the straight keel bones and the sharpness of the nails that adequate quantities were present. Some tryptophan was evidently destroyed but no lysine was lost. The data indicate that all of the nutrients studied were present after sterilization in quantities adequate to meet the requirements of the chick (Bird, '47). This was substantiated by the growth and appearance of the control chicks fed the autoclaved diet during this study. Vitamin B_{12} appeared to be present in adequate quantities except in one experiment (not reported) wherein hot alcohol-extracted casein was used.

TABLE 7

Stability of nutralites during sterilization and storage

NUTRALITE	DIET NO. 56227			SUPPLEMENT [1]		
	Fresh	Autoclaved	Stored [2]	No.	Theoretical	Found
Thiamine (μg/gm)	13.0	2.0	2.0	2945	400	246
Riboflavin (μg/gm)	15.5	9.5	10.0	108A	500	510
Nicotinamide (μg/gm)	47.0	38.8	34.0	2945	800	680
Pantothenic acid (μg/gm)	40.0	27.0	33.0			
Pyridoxine (μg/gm)	10.7	8.3	8.3	2945	1,100	840
Biotin (μg/gm)	0.45	0.46	0.38			
Choline (mg/gm)	1.45	1.28	1.16			
Folic acid (μg/gm)	3.3	1.2	1.1			
Vitamin A (U.S.P. units/gm)	21.7	17.7	14.3	Concentrate	78.0	72.0
Vitamin E (mg/gm)	0.52	0.52		Oil supp.	16,000	13,280
Vitamin K (mg/gm)	0.05		0.031			
Lysine (mg/gm)	25.0	29.0	30.0			
Tryptophan (mg/gm)	4.0	3.7	2.8			

[1] The supplements used are: no. 2945, B complex in water; 108A, thiamine in weak acid solution; norite eluate, see table 1, footnote 4; oil supplement, a mixture of vitamins A, D, E, and K in corn oil.

[2] Autoclaved, then stored one week at room temperature.

DISCUSSION

The fact that chicks reared in an environment which excludes all other demonstrable living organisms grow and develop in a manner comparable to contaminated control chicks reared under normal laboratory conditions shows that the diet is basically complete. Thus, the intestinal microorganisms contribute little, if any, important unidentified nutrients not present in the diet under the conditions described. It is possible that the diets carried significant quantities of an unknown factor or factors, or that newly hatched chicks possess a reserve of some factor adequate for the first few weeks of life. Also, criteria of health other than the gross observations reported here may reveal unrecognized deficiencies. Thus the work presented herein delineates the problem but does not treat it exhaustively.

The present experiments show that germ-free chicks may be obtained routinely, reared satisfactorily on experimental diets, and handled neatly for experimental work. The methods developed provide acceptable standard conditions for carrying out planned research programs with germ-free chicks in nutrition and in other fields of biological experimentation. The occasional increased growth rate of germ-free chicks over that of control chicks fed the same diet raises the question of whether the net effect of the microorganisms in the gastrointestinal tract of the control birds may be detrimental. It would be interesting to learn how much the myriad intestinal microorganisms decrease food efficiency by direct competition with the host for essential nutrients or energy under adverse conditions, such as the suboptimum supply of a given vitamin.

The results of the present study show that the general nutritional pattern of germ-free chicks is similar to that of laboratory-reared chicks, and offer a measure of progress in the field of nutrition. Previously the relative completeness of our knowledge has been indeterminate because of uncertainty about the contribution of the intestinal microorganisms. The finding that conventional experimental diets already de-

veloped allow good growth and general well-being without contribution from living microoganisms is assurance that the foundations of nutrition will be changed more by addition than by revision. While much remains to be done with different types of diets, the use of different animal species, other criteria of health, the balance of nutrients, growth stimulants, and so forth, the search for new vitamins appears to be forming not a straight line or spiral ad infinitum, but rather a circle or a unit of knowledge whose general outline is already known.

SUMMARY

1. Chicks reared for short periods of time (4 to 8 weeks) on experimental diets in the complete absence of other demonstrable living forms grew and developed as well as control chicks fed the same diets.

2. The addition of sources of unidentified vitamins did not significantly increase the growth rates of germ-free chicks.

3. A study of the stability of vitamins and amino acids in the diet during steam sterilization and subsequent storage indicated that thiamine was the only nutrient seriously affected.

4. Large quantities of vitamins were found in the cecal contents of germ-free as well as of control chicks, showing that vitamins found in the lower intestinal tract are not necessarily of bacterial origin.

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NIACIN DEFICIENCY AND ENTERITIS IN GROWING PIGS[1]

WISE BURROUGHS, B. H. EDGINGTON, W. L. ROBISON AND R. M. BETHKE

Department of Animal Science, Ohio Agricultural Experiment Station, Wooster

ONE FIGURE

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Chick et al. ('38) observed niacin deficiency in pigs while feeding a blacktongue diet made up chiefly of natural feeds. Enteritis was observed on this diet and attention was called to its similarity to the American condition known as necrotic enteritis in swine. No *Salmonella cholersuis* organisms, however, could be isolated. Madison et al. ('38) observed a diarrhetic condition in pigs reared on farm feeds, which was alleviated by niacin supplements. Davis and others ('40b) encountered enteritis in pigs while feeding single grains, corn in particular, along with liquid skim milk; the addition of niacin benefited these pigs. Later investigations by Davis and Freeman ('40a) and Davis et al. ('43) met with mixed success in attempting to correlate niacin deficiency with enteritis. Edgington et al. ('42), using natural feeds, failed to observe any protective influence of niacin against *S. cholersuis* infection. More recently, Luecke et al. ('47a, b) produced a niacin deficiency in pigs fed a corn diet containing limited soybean oil meal. Powick et al. ('47b) failed to obtain niacin deficiency symptoms in pigs while using two basal rations made up chiefly of corn.

[1] Published with the approval of the Director of the Ohio Agricultural Experiment Station.

Some of the apparent discrepancies in producing niacin deficiency and enteritis in pigs fed natural feeds may be due to the variable requirement of pigs for this vitamin and the variable niacin content of corn. Burkholder and associates ('44) and Hunt et al. ('47) found considerable variation in the latter. Wintrobe et al. ('45) pointed to an increased need for niacin with decreased levels of ration protein. This influence of protein was not fully confirmed by Powick et al. ('47a) or Luecke and co-workers ('47b). Both groups of investigators, however (Luecke et al., '47b; Powick et al., '48), found tryptophan effective in reducing or replacing the niacin needs of pigs.

The purpose of the present paper is to present further observations on niacin deficiency and enteritis in young pigs fed rations made up largely of natural feeds.

EXPERIMENTAL

Experiment 1

The basal ration used in the initial experiment was essentially the same as that used by Chick and associates ('38). It contained, on a percentage basis, ground yellow corn 80, cowpeas 10.5, commercial casein 6.5, complex mineral mixture 2.5, and vitamin A and D feeding oil 0.5. Sixteen weanling pigs averaging 25 lb. in live weight were divided into two groups of 8 each. One group received the basal ration and the other the basal ration plus 60 mg of niacin per pig daily. The pigs were individually fed all the feed they would consume for a period of 10 weeks.

The results of this test were negative throughout. The pigs that received the basal ration grew just as well (average daily gain 0.70 lb.) as those that received added niacin. No scouring was noted in any of the pigs. Blood serum, protein and hemoglobin determinations made at biweekly intervals were within normal ranges throughout the test.

The niacin content of the basal ration was 15.2 μg per gram as determined microbiologically. Comparing this ration with

the one used by Chick et al. ('38), it is possible that the English ration may have had a lower niacin content, since it contained corn grits and purified casein in place of ground yellow corn and commercial casein. Also, body storage of niacin may have been greater in our pigs since they were pasture-reared prior to being placed on experiment.

Experiment 2

Two rations low in niacin were used in the second experiment. One was a modification of the English ration used in the first experiment. It contained, in per cents, ground yellow corn 60, corn starch 20, ground cowpeas 10.5, commercial casein 6.5, complex mineral mixture 2.5, vitamin A and D feeding oil 0.5, and adequate supplements of thiamine, riboflavin, calcium pantothenate and pyridoxine. The other ration was similar to that employed by Luecke et al. ('47a). It contained, in parts by weight, ground yellow corn 88.5, soybean oil meal 5.5, commercial casein 3.5, complex mineral mixture 2.5, vitamin A and D feeding oil 0.5, and supplements of the above 4 B vitamins. Each of the rations contained 13% total protein. The cowpea ration contained 10.0 μg of niacin per gram and the soybean oil meal ration 12.5 μg per gram.

Eighteen pasture-reared pigs approximately 10 weeks old and averaging 31 lb. in live weight were divided into three groups of 6 pigs each. One group was placed on each of the two rations, while the third group received the modified English ration plus 60 mg of niacin per pig daily. Each pig was individually fed for 16 weeks and observed daily for symptoms of diarrhea.

Blood hemoglobin was determined at 4-week intervals on all pigs, with determinations more often in severely affected pigs. Total red and white blood cell, as well as differential white cell, counts were made.

Growth on the modified English ration was poor, averaging only 0.40 lb. per pig daily (table 1). The addition of

niacin to this ration increased the average daily gain to 0.92 lb. The ration containing soybean oil meal produced an average daily gain of 0.87 lb., which was almost equal to that of the niacin-supplemented group. Feed consumption in the three lots paralleled the rates of gain.

The incidence of scours was highest in the pigs fed the unsupplemented cowpea ration. Some scouring was noted on the soybean oil meal ration but little or no diarrhea (0.7%) occurred in the pigs fed the cowpea ration supplemented with niacin (table 1). Based upon total pig days in a given lot,

TABLE 1

Results of second experiment comparing two niacin-low rations with a niacin-supplemented ration fed to weanling pigs

FACTORS STUDIED	LOT DESCRIPTION		
	Cowpea basal ration	Cowpea basal + niacin	Soybean oil meal basal
Average initial live weight (lb.)	31	31	31
Average daily live weight gain, 16 weeks (lb.)	0.4	0.9	0.9
Average daily ration consumed (lb.)	2.0	3.2	3.2
Diarrhea occurrence (% of total pig-days)	21	1	12
Blood analyses at end of experiment:			
Hemoglobin (gm per 100 ml blood)	9.4	13.4	12.0
Red cell count (× 1000)	6360	7940	7526
White cell count (× 1000)	36	20	31

the percentage of time the pigs scoured in the three lots was 21, 12, and 1, respectively. The incidence of scours in a given lot varied considerably. The initial onset of scours in some pigs began after three weeks on test, while other pigs began to scour at a much later time. Conversely, some pigs in the same lots had well formed stools at all times. The intensity of the diarrhea also varied, the more severely affected animals having very liquid feces, sometimes highly colored, and showing the presence of blood. Examinations of the fecal matter for *S. cholersuis* were negative.

Blood hemoglobin in the niacin-supplemented lot and in the soybean oil meal lot was maintained in the range of from

12 to 13 gm per 100 gm of blood (table 1). Average hemoglobin levels in the cowpea basal lot dropped at each succeeding 4-week period to a final value of 9 gm per 100 gm of blood. The two most anemic pigs in this lot were the two with the severest diarrhea. Folic acid supplements were given these two pigs between the 12th and 16th weeks with little or no effect upon the existing anemia.

The red blood cell count (table 1) averaged slightly more than 6 million in the low hemoglobin lot, cowpea basal, and approximately 8 million in the other two lots. Nucleated red cells were present in the lot showing anemia (cowpea basal). The white cell count was normal (average 20,000) in the niacin-supplemented lot. Average values in the non-supplemented lots were 31,000 in the soybean oil meal and 36,000 in the cowpea basal groups. Differential white cell counts fell within the normal range in all lots.

At the close of the test all pigs were submitted for post mortem examination. The pigs that had received added niacin appeared normal throughout. The pigs fed the two low niacin rations (cowpea and soybean oil meal basals) showed varying degrees of disturbances in the large bowel, with some individuals appearing quite normal. In the more severely affected animals the entire colon exhibited a mild inflamation and necrosis which appeared chronic in nature. The intestinal wall was slightly thickened, friable, and in some cases was "corky" or "pulpy" in appearance. The mucosa lining the colon was usually discolored and many times fecal material would adhere so firmly to the intestinal wall that it could not be washed off with water (see fig. 1). Attempts to remove this material usually resulted in denuding the mucosa. Small elevated ulcerated areas 0.3 to 1.0 cm in diameter were found in the more severely affected animals. In the less severely affected pigs these areas could sometimes be located with difficulty, suggesting that healing action had taken place or that the ulcer was just beginning to form.

The mesentery lymph glands were usually enlarged where the disturbance in the large bowel was marked. In a few

cases these glands were edematous. Attempts to culture *S. cholersuis* organisms from these glands were negative. No further abnormalities were noted with regularity in the pigs fed the low niacin rations.

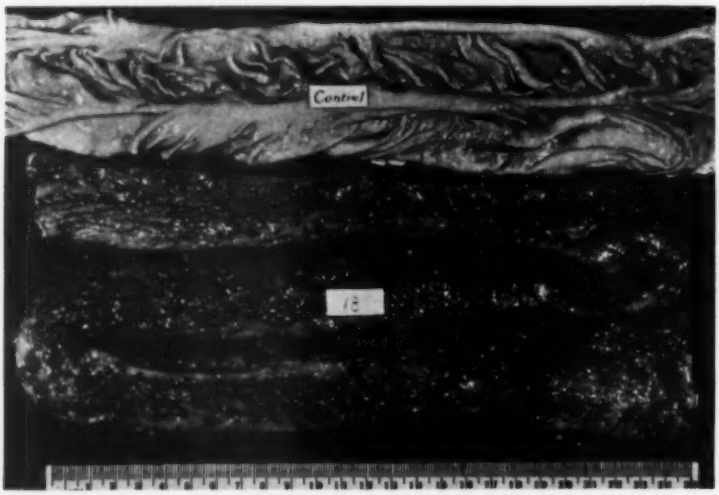

Fig. 1 Colon of a niacin-deficient pig, no. 18, compared with a normal colon from a control pig receiving niacin supplements. Note darkened areas representing necrotic areas to which fecal material is firmly attached.

Experiment 3

The third feeding trial was patterned after the previous experiment but designed for the purpose of producing either more severe niacin deficiency symptoms or greater uniformity of symptoms between pigs in a given lot. The niacin content of the two basal rations was reduced by adding starch to each. Pigs reared indoors were used in all lots except one in order to minimize the carry-over or body storage influence which is sometimes credited to previous pasture feeding. One lot of pasture-reared pigs was included and compared directly with similar pigs reared indoors. The rations and

lots as fed are presented in table 2. Each ration contained 12% protein. The niacin content of basal rations 1 and 2 was almost identical, being 8.8 and 8.5 μg per gram of feed, respectively.

The pigs were individually fed and weighed weekly, and daily observations were made to note the incidence of scours.

TABLE 2

Ration fed in third experiment

FEED INGREDIENTS	BASAL RATION [1]	
	1	2
Ground yellow corn, lb.	50.0	50.0
Corn starch, lb.	30.0	35.0
Ground cowpeas, lb.	10.5	
Soybean oil meal, lb.	...	5.5
Commercial casein, lb.	6.5	6.5
Bone meal, lb.	1.0	1.0
Limestone, lb.	1.0	1.0
Iodized salt, lb.	0.5	0.5
A and D feeding oil, gm	40.0	40.0
Ferrous sulfate, gm	10.0	10.0
Manganese sulfate, gm	5.0	5.0
Copper sulfate, gm	0.5	0.5
Cobalt chloride, gm	0.2	0.2
Thiamine, mg	100.0	100.0
Riboflavin, mg	150.0	150.0
Calcium pantothenate, mg	740.0	740.0
Pyridoxine, mg	120.0	120.0
Niacin	....	....

[1] Lots 1 and 2 were fed basal ration 1. The 6 pigs in lot 1 were pasture-reared, while the 6 pigs in lot 2 were reared indoors. Lot 3 consisted of 6 pigs reared indoors which were fed basal ration 1 plus 60 mg niacin per pig daily. Lot 4 consisted of 6 pigs reared indoors which received basal ration 2 without niacin supplementation.

Blood hemoglobin was determined at periodic intervals. A few of the pigs exhibiting niacin deficiency were placed on recovery trials at the end of the regular feeding period.

The gains made during the 12-week feeding period were very poor for the pigs reared indoors and receiving the two low niacin rations (table 3). The average daily gains were

0.25 and 0.12 lb. for the lot 2 and lot 4 pigs, respectively. The pigs reared indoors and fed niacin plus the first basal ration gained substantially more (0.80 lb. daily) than similar pigs without supplemental niacin. Comparing the average daily gains made by lots 1 and 2 — 0.49 and 0.25 lb., respectively — it is evident that the pigs reared on pasture were superior to those reared indoors when fed the niacin-low ration following weaning. Presumably this represents a body storage or carry-over influence of nutrients obtained from pasture feeding.

TABLE 3

Results of third experiment comparing niacin-low rations with a niacin-supplemented ration for young pigs

FACTORS STUDIED	LOT NO. AND DESCRIPTION			
	1 Cowpea ration, pigs pasture-reared	2 Cowpea ration, pigs reared indoors	3 Cowpea + niacin, pigs reared indoors	4 Soybean ration, pigs reared indoors
Average initial live weight (lb.)	37	36	37	35
Average daily live weight gain, 12 weeks (lb.)	0.5	0.3	0.8	0.1
Average daily ration consumed (lb.)	2.0	1.6	2.9	1.2
Diarrhea (% of total pig-days between 4th and 12th weeks)	44	37	0	79
Blood hemoglobin at end of experiment (grams per 100 ml blood)	11.8	9.4	11.8	9.4

The degree of scours (table 3) in the various lots paralleled the poorness of gains in each lot. No diarrhea was noted in the best-gaining lot fed niacin. The percentage of scours in lots 2 and 4 receiving the two basal rations with no niacin additions was 37 and 79, respectively, from the 4th to 12th week on experiment. The pasture-reared pigs scoured as much as the pigs reared indoors, despite the fact that the former group gained in body weight somewhat faster. The comparative figure for percentage of scours in the pasture-reared pigs, lot 1, was 44.

Blood hemoglobin in lots 2 and 4 (basal rations) dropped from an initial value of 12 to 13 gm% to an average value of

9 gm. The pigs fed niacin (lot 3) and the pasture-reared pigs (lot 1) maintained normal blood hemoglobin levels throughout the 12-week feeding period.

Four severely deficient pigs were subjected to recovery trials. Each of these pigs was given 100 mg of niacin daily, either directly in the feed or by drenching. These pigs had scoured almost continuously from the 4th to the 12th week on one of the low niacin rations. At this time two of the pigs were eating meagerly and the other two had refused feed for almost a week.

Following niacin supplementation, appetite improved slightly the first three days and feed consumption increased markedly throughout the remainder of the 4-week period. At the end of the period each pig was consuming almost double the amount of feed considered a full allotment for pigs weighing 50 to 60 lb. Diarrhea cleared up in each pig within 11 days after niacin feeding was begun.

Sulfa drugs were given by capsule to three pigs which were scouring on the low niacin rations, in an attempt to alleviate the diarrhea. Sulfathalidine was tried first, 8 gm per pig being given the first day and 4 gm the following three days. Feed consumption was depressed somewhat but no change was noted in the incidence of scours. Three days later the same pigs were similarly treated with sulfasuxidine. The diarrhea was not altered by this second treatment.

DISCUSSION

There can be little doubt but that niacin deficiency was produced in two of the three experiments in which two different basal rations were used. The deficiency symptoms noted were similar to those observed by Luecke et al. ('47a) and Powick et al. ('47a). These include poor appetite, slow growth, rough haircoat, anemia, diarrhea, and, upon post mortem examination, disturbances in the large bowel.

The diarrhea encountered while feeding low niacin rations was not associated with *S. cholersuis* infection and very probably was not related to any specific infection of the

large bowel. *S. cholersuis* organisms were not recovered through culturing the feces or tissues of severely affected pigs. The body temperatures of pigs with scours were normal at all times. Further evidence that the diarrhea produced was of nutritional origin rather than the result of an infectious agent was the alleviation of symptoms with niacin supplementation as contrasted with the negative results obtained with two sulfa drugs.

The failure to produce niacin deficiency in the first experiment may have been due to an adequate amount of niacin in the basal ration. This ration contained 15.2 μg of niacin per gram. The basal rations producing niacin deficiencies in the second experiment contained 10.0 and 12.7 μg and in the third experiment 8.8 and 8.5 μg of niacin per gram of ration. According to Powick et al. ('47b) the niacin needs of young pigs are met by 0.6 to 1.0 mg of niacin per kilogram of live weight; the basal ration in the first experiment would supply slightly more than 0.6 mg. Similarly, in the second and third experiments the basal rations would supply only about half to two-thirds the amount of niacin needed by young pigs, as judged from the results of Powick and associates ('47a, b).

Despite the general agreement between the present results and those of Powick and associates ('47b) as to the practical requirement for niacin, it is difficult at this time to estimate the likelihood of encountering niacin deficiency in pigs under all feeding conditions. As was mentioned earlier, the niacin requirement of pigs is influenced by the amount of protein or tryptophan or both in the ration. In addition to this, there is reason to believe that there may be other factors influencing the niacin needs of pigs. For example, attention has been called to the wide variation in severity of symptoms noted between pigs in the same lot with similar breeding and environmental backgrounds. It is possible that the low niacin rations used in these and other experiments were not entirely adequate in other members of the vitamin B

complex. The extent to which these factors, as well as tryptophan and other protein components, influence niacin requirements needs further study.

SUMMARY

Weanling pigs were individually fed rations low in niacin during three feeding experiments. Niacin deficiency was produced with two rations whose major ingredients were corn, starch, casein and either cowpeas or soybean oil meal. The niacin content of these rations was less than 15 μg per gram. The principal deficiency symptoms noted in the pigs were poor appetite, slow growth, roughened haircoat, diarrhea, anemia, and disturbances in the large bowel.

Pigs receiving similar rations to which were added 60 mg of niacin daily, grew and developed normally. Deficient pigs also responded to niacin feeding during recovery trials.

The diarrhea produced was not alleviated by sulfathalidine or sulfasuxidine. It at no time produced body temperature elevations similar to those encountered in diarrheas caused by infectious agents.

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ENAMEL EROSIVE PROPERTIES OF FRUITS AND FRUIT JUICES[1]

CAREY D. MILLER

Foods and Nutrition Department, Agricultural Experiment Station, University of Hawaii, Honolulu

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INTRODUCTION

A number of investigators (McClure, '43; Gortner, Restarski, Bieri and McCay, '45; Bieri, McCay, Restarski and Gortner, '46; and Wynn and Haldi, '48) have shown that acid beverages, natural or synthetic, when fed to rats cause destruction of the lingual enamel of the molar teeth. No one seems to have tested the reaction to acid fruits themselves, nor compared their enamel erosive properties with those of the juices made from them.

We had assumed that acid fruits have an effect similar to that of the juices, but this appears not to be true. The present paper presents data on the enamel erosive properties of 5 fruits and the juices made from them.

PROCEDURE

Animals and their teeth

Young female albino rats from our colony were weaned at three weeks of age, placed in separate cages, and continued on the stock diet until they weighed approximately 155 gm at 7 to 8 weeks of age. (All males were used for vitamin A assay.) Free access to food and water was permitted when the supplements were fed. In a few cases, if the full supplement was not readily taken, the food cup or the water was

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removed from the cage for a part of the day or for overnight.

Insofar as possible, matched litter mates were used for testing the fruits and the juices prepared from them. All supplements were fed for 5 days (Monday to Friday) and on the 6th day the rats were killed. One rat out of 10 was used as a control.

The jaws and teeth were removed, prepared and examined by the method recommended by Restarski, Gortner and McCay ('45) with some modifications. The heads of the rats were placed in individual beakers with the mouth upward and, to stain the teeth, 0.5 ml of 0.125% alizarin red in a 20 to 25% alcoholic solution was placed in each mouth before autoclaving. The stain aids greatly in differentiating the degree of etching. The first evidence of any effect upon the teeth is that the enamel takes the stain, whereas the enamel of the teeth of controls and of those animals given certain supplements show little or no stain.

Only the molars of the mandible were scored because, in comparison, the upper molars were very slightly affected. With a supplementation period of 5 days no rats presented the most severe destruction, which Restarski et al. ('45) rate as 6. As in the report by Wynn and Haldi ('48), high polish of lingual enamel, slight etching, mild destruction, moderate destruction and severe destruction were graded, respectively, 1, 2, 3, 4 and 5.

Since only one person examined the teeth, to obtain completely objective scores an assistant handed the teeth to the author who, without knowing the supplement fed, announced the score, which was recorded by the assistant. The teeth were rechecked on a different day and if there was lack of agreement with the previous scores, they were scored a third time.

Fruits and fruit juices

Preparation. Some fruits and juices were fed in the fresh state, others were cooked. Grapefruit were peeled and the

membranes removed from each section. One-half of each fruit, divided from stem to blossom end, was used for fresh fruit and the other for juice, which was made by squeezing a known weight of fruit through two thicknesses of cheesecloth.

The outer heavy rind of guavas, here referred to as the "shells," was used both in the raw and cooked states. The guavas were cut in half and the seedy portion removed and treated separately. One-half of each shell was used for the composite raw sample, the other half for juice. The halves used for juice were sliced and weighed, a known weight of water was added and, after being cooked for 15 minutes, they were strained in a jelly bag. After ceasing to drip the bag was squeezed gently to remove the maximum quantity of juice, which was weighed, measured and stored in a refrigerator until used.

For cooked guavas the finely chopped shells were placed in a beaker, covered and heated in a pressure cooker for 5 minutes at 15 lb. pressure.

The seedy portion from each guava was divided into two lots. One composite was put through a strainer to make fresh, uncooked puree; the other was used to prepare the cooked juice in a manner similar to that used in obtaining the "juice" from the shells.

Java plums *(Eugenia cumini)* from the same picking were divided into two equal lots. The flesh was cut from the seeds and fed as fresh fruit. After being weighed the corresponding lot was covered with water, cooked until tender, and strained as were the guavas. Seeds were recovered and weighed so that the amount of juice equivalent to the edible portion could be calculated.

The half-ripe mangoes were peeled and the edible portion fed raw without sugar. The rats ate the half-ripe mango with the same gusto as do children in Hawaii. Green or half-ripe mangoes contain starch and when they are cooked with water they form a thick colloidal mass from which it would be extremely difficult to get any juice. Therefore, a known weight

of half-ripe mangoes was cut in small pieces, water was added and the resulting material mixed for about one minute in a Waring Blendor, strained in a cloth and the resulting "juice" measured.

Only fresh, high quality pineapple of the Smooth Cayenne variety was used. The opposite quarters of each fruit were cut in chunks, weighed and squeezed to produce a measured amount of juice. The remaining two quarters were chopped and fed as fresh fruit.

Ten to 15% of sugar was added to most of the fruits and juices to induce the rats to eat them more readily. The raw half-ripe mangoes were fed without added sugar and the mango juice with 5% sugar. The weight of the fruits and volume of juices given in the table are for the mixture of fruit and sugar as fed. For example, 20 gm guava shells (table 1) means 17 gm of edible guava plus 3 gm of sugar. To the amount of juice calculated to be equivalent to 17 gm of fresh guava, 15% of sugar was added.

Glass drinking tubes having a J shape were used for the fruit juices, and special supplement cups for the fruits.

pH and titratable acidity of fruits and juices. The methods used were essentially those recommended by the A. O. A. C. ('45). The determinations of pH and titratable acidity were made on the products containing sugar as fed.

Fruits. Fifteen grams were weighed in a 250-ml beaker. The pH was read on the industrial model Beckman pH meter. With the exception of pineapple, the mixtures were stirred in a Waring Blendor, distilled water added to make a total volume of 150 ml, and the whole titrated to pH 8.1 with approximately 0.1 N NaOH. The results were converted to exactly 0.1 N NaOH and the amount of 0.1 N acid per 100 gm of fruit was calculated.

Juices. Fifteen milliliters were pipetted into a 250-ml beaker, the pH read and the volume made up to 150 ml with distilled water. The mixture was titrated to pH 8.1 and the milliliters of 0.1 N acid per 100 ml of juice were calculated.

RESULTS AND DISCUSSION

Data pertinent to the experiments are summarized in table 1.

There are practically no differences between the pH values for the fruits themselves and for their juices.

The titratable acidities of the expressed grapefruit and pineapple juices are markedly higher than those of the fruits. The acidity for the pineapple probably would have been somewhat higher if the fruit had been more thoroughly mixed with the distilled water in the Waring Blendor. The Java plum juice has a titratable acidity only slightly higher than that of the fruit, and the cooked guava juice a lower titratable acidity than the raw or cooked fruit.

Through an oversight the pH and titratable acidity of the half-ripe mangoes fed were not determined. Later two other lots of mangoes, one green and one half-ripe, showed the pHs of the fruit and the juice to be similar, but the titratable acidities for the mangoes were greater than were those for the juices. For green mangoes the figures for fruit and juice, respectively, were 124 and 91 and for half-ripe mangoes 97 and 50. As with guavas, the titratable acidity of the fruit is greater than that of the juice.

The pulp from the seedy portion of guavas and the juice made from it always have a greater acidity than the guava shells and their juice. However, the difference is not always as great as might be judged from the figures in table 1. This pulp and its juice were made from guavas obtained later in the season than the shells which were first fed. The pH and titratable acidity were determined for shells and juice of the same guavas which were used for pulp. The pH of both shells and juice was 3.8; the titratable acidities were 243 for shells and 129 for juice.

The scores for the controls given stock diet and water were zero. It may be noted (table 1) that the average molar scores for all the sweetened fruits fed at a level of 20 gm per day for 5 days, except grapefruit, were 0.7 or less. This usually

TABLE 1

In vivo decalcifying effect of acid fruits and fruit juices on lower molar teeth of rats

FRUIT OR JUICE	pH	TITRATABLE ACIDITY	NO. OF RATS	DAILY SUPPLEMENT		AVERAGE MOLAR SCORES	
				Fruit	Juice	Fruit	Fruit juices
		ml		*gm*	*ml*		
Controls (no supplements)			12			0	
Grapefruit	3.9	197	6	20		2.0	
Grapefruit Juice ≏ 20 gm fruit	3.8	228	6		13		4.6
Grapefruit Juice ≏ 10 gm fruit	3.8	250	5		6.5		3.5
Guava shells, raw	4.1	167	9	20		0.7	
Guava shells, cooked	3.9	252	5	20		0.1	
Guava shells Juice ≏ 20 gm fruit	4.2	110	6		21		4.6
Guava shells Juice ≏ 10 gm fruit	4.2	118	5		10.5		3.9
Guava shells Juice diluted ≏ 10 gm fruit (10.5 ml guava juice + 9.5 ml water)	4.2	61	5		20		4.3
Guava pulp, raw	3.6	309	5	17		0.6	
Guava pulp Juice ≏ 8.5 gm pulp	3.6	215	5		15		4.0
Java plum	3.9	184	5	20		0.4	
Java plum Juice ≏ 13.3 gm fruit	4.0	204	5		12.5		4.8
Java plum Juice ≏ 10 gm fruit	3.6	206	5		9.5		4.1
Mango (half ripe), raw			5	20		0.2	
Mango Juice ≏ 20 gm fruit	4.2	146	5		20		3.2
Mango sauce, cooked (from green and half-ripe mangoes)	4.5	72	5	20		0.6	
Pineapple	4.0	96	8	20		0.09	
Pineapple Juice ≏ 20 gm fruit	4.0	180	6		11		2.7

meant that out of 5 rats, two or three showed no etching of the molars and two or three showed some slight effect — often on only one side of the mandible, sometimes on only one or two of the teeth.

It was impossible to prepare grapefruit without a juicy exudate which the rats consumed along with the fruit itself. The consumption of the juice may have accounted for the higher molar score of grapefruit.

Average molar scores of 4.6, 4.6, and 4.8 for the grapefruit juice, guava juice, and Java plum juice, respectively, indicate severe destruction of lingual enamel in comparison with the slight effect of the fruits themselves.

It may also be noted that even when the rats were given juice supplements equivalent to half the quantity of fruit fed, the scores for grapefruit, guava and Java plum juices were 3.5 to 4.1. Even when guava juice equivalent to half the fruit supplement was diluted by half with water, the molar score was high (4.3).

Like the guava shells, the pulp gave a low average molar score (0.6), whereas juice equivalent to half the weight of the pulp gave a high average score (4.0), just as did the juice made from the shells.

The enamel erosive properties of pineapple and pineapple juice appear to be definitely less than those of the other fruits and juices tested. Gortner et al. ('46) have shown that small amounts of oxalate in an acid beverage tend to protect the tooth enamel, but Clark ('39) found the quantity of oxalate in pineapple to be extremely small. It may nevertheless have some protective effect.

As yet we have no satistfactory explanation for some of the results shown in table 1. Muller and Gortner ('49) state that "The degree to which acid solutions attack the teeth is a function of the titratable acidity of the solutions." If that is true, why do 20 ml of diluted guava juice with a pH of 4.2 but a titratable acidity of only 61 have almost as great an enamel erosive effect as an equal amount of undiluted guava juice with the same pH but a titratable acidity of 110?

To determine if some heat labile substance in the fresh fruit might be the protective factor, guava shells prepared as for feeding were heated as described above and then fed. As may be seen in table 1, the cooked guava shells gave an average molar score slightly lower than that obtained with raw ones.

Why fruits and fruit juices affect the molar teeth of rats differently remains to be determined. Some possible explanations may be: (1) the greater buffer action of the fruits themselves; (2) some principle which the fruits contain but the juices do not; (3) some constituent formed in the juice when it is expressed from the fruit; (4) a difference in the physical contact with the teeth when the two are consumed; or (5) some combination of these factors. That a marked difference exists is clearly demonstrated by the data presented.

Preliminary experiments with Boysenberries and juice and with tamarind and juice indicate results similar to those detailed in this paper. It seems safe, therefore, to postulate that acid fruits themselves may have little or no enamel erosive effects, whereas the juices made from them are certain to have this effect in varying degree.

SUMMARY AND CONCLUSIONS

1. Five tropical and semi-tropical fruits — namely grapefruit, guava, Java plum, mango, and pineapple — and juices prepared from them have been fed to standard rats to determine their enamel erosive properties.

2. The pH and titratable acidity were determined for each lot of sweetened fruit and fruit juice used.

3. Quantities of juice equivalent to the daily fruit supplement had three to 10 times the enamel erosive properties of the fruits themselves.

4. No adequate explanation for the different effect of fruit and fruit juice can be offered at the present time, but it appears not to be related to the titratable acidity of the two.

5. Data given for 5 fruits of widely different species and preliminary experiments with two other fruits appear to jus-

tify the postulate that acid fruits generally have a slight enamel erosive effect, in contrast to the marked effect of the juices made from them.

ACKNOWLEDGMENTS

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VITAMIN B_{12} CONCENTRATES IN THE NUTRITION OF THE MATURE DOMESTIC FOWL[1]

ORLANDO OLCESE, J. R. COUCH AND CARL M. LYMAN
Departments of Biochemistry and Nutrition and Poultry Husbandry, Agricultural and Mechanical College of Texas, and Texas Agricultural Experiment Station, College Station

FIVE FIGURES

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Cravens and Halpin ('49) reported that an unidentified factor is required by the hen for egg production and hatchability, and stated further that liver "L" and fish solubles are good sources of the factor in question. Different authors have presented evidence for the existence of unidentified factors that improved growth performances in several other species when purified diets containing all known B vitamins were fed. Cary et al. ('46) and Zucker and Zucker ('48) showed that an unidentified factor is essential for rat growth which apparently is identical to the growth factor from cow manure required by the chick. Kunkel et al. ('48) found that liver contains an unidentified factor essential for a maximum rate of growth in the rabbit. Liver concentrates have also been shown to improve the growth performance of monkeys (Cooperman et al., '46; Ruegamer et al., '47) and of the mink and fox (Schaefer, Whitehair and Elvehjem, '48b; Schaefer, Tove, Whitehair and Elvehjem, '48a) when purified diets containing all known vitamins are fed.

Recently vitamin B_{12} was obtained in crystalline form (Rickes et al., '48). Since then, reports have appeared in

[1] Supported in part by a grant from Lederle Laboratories, Pearl River, New York.

the literature pointing to the ability of vitamin B_{12} to produce normal growth in chicks. Ott, Rickes and Wood ('48) reported that amounts as small as 6 μg of vitamin B_{12} per kilogram of diet stimulated the growth of chicks as much as dietary supplements consisting of crude sources of this factor. Nichol, Dietrich, Cravens and Elvehjem ('49) have reported that vitamin B_{12} is highly active in stimulating the growth of hyperthyroid chicks. They concluded that vitamin B_{12} could replace the animal protein factor activity of condensed fish solubles and injectable liver preparations. Stokstad et al. ('49) used chicks from depleted hens and found that a level of 15 μg per kilogram of diet or 0.3 μg injected intramuscularly each week supplied the vitamin B_{12} requirement of the chicks, but maximum growth was not obtained until certain other supplements were added. Yeast, alfalfa meal, distillers' solubles or fish solubles had little or no effect in this regard.

The experiments reported in this paper were conducted to determine the effectiveness of vitamin B_{12} concentrates in maintaining production, health and hatchability in the mature fowl.

EXPERIMENTAL

The management and general experimental procedure were the same as those previously reported by Couch et al. ('48).

The composition of the diets is shown in table 1. Soybean protein, supplemented with glycine and methionine, was used as a source of protein. Soybean protein was used by Neumann, Krider and Johnson ('48) in a study of the unidentified growth factors needed for optimum growth in newborn pigs. The carbohydrates used were sucrose and starch. Crystalline B vitamins were added in amounts which were believed to meet the requirements of the pullets. Animal protein factor (APF) concentrates were used as a source of vitamin B_{12}. Three sources of the latter were used;[2] these three supple-

[2] One was concentrate 1 of Merck and Co.; the other two were supplied by Lederle Laboratories under the numbers R-1 and 197 B.

ments were analyzed for vitamin B_{12} activity by a modification of the microbiological method of Skeggs et al. ('48), using *Lactobacillus leichmannii* (American Type Culture Collection 4797) as the test organism. APF concentrate 1 was found to contain about 3.6 μg of apparent vitamin B_{12} per gram; concentrate R-1 was found to contain about 1.0 μg per

TABLE 1

Composition of diets [1]

	A-1	A-2	A-3	A-4	A-5	A-6	A-11	A-12	A-13	A-14
Sucrose	68	68	68	68			68	68	68	68
Corn starch					68	68				
Soybean protein [2]	22	22	22	22	22	22	22	22	22	22
Soybean oil	3	3	3	3	3	3	3	3	3	3
Fish oil (3,000 A — 400 D)	2	2	2	2	2	2	2	2	2	2
Salts IV	5	5	5	5	5	5	5	5	5	5
Supplements (gm/kg)										
Merck B_{12} conc. no. 1							5	10		
Lederle APF conc. R-1	0	2.5	5	10	0	2.5			15	
Lederle APF conc. 197 B										10

[1] The following amounts of vitamins and amino acids were added per kilogram of diet: Thiamine hydrochloride 4 mg, riboflavin 6 mg, calcium pantothenate 15 mg, niacin 100 mg, pyridoxine hydrochloride 4 mg, 2 methyl-1,4-naphthoquinone 0.5 mg, alpha-tocopherol 3 mg, pteryolglutamic acid 2 mg, biotin 0.2 mg, choline chloride 2000 mg, methionine 7.5 gm, glycine 4 gm.

[2] Type M, Buckeye Cotton Oil Co., Cincinnati, Ohio.

gram; and concentrate 197 B contained about 0.5 μg per gram. The latter value is within the range of that reported by Stokstad et al. ('49) for such a concentrate using *L. leichmannii* 313 (American Type Culture Collection). Oyster shells and tap water were supplied ad libitum.

Eggs were set aside during the first two days of each week and also at the end of each experiment to be assayed for vitamin B_{12} activity and for an unknown factor required by *Leuconostoc citrovorum* 8081. Assays were run on egg yolks only and weekly samples of eggs from each group were pooled. Liberation of the vitamins was accomplished by incubation of the samples with pancreatin at pH 7.8 and 38°C. for 24 hours. Following this, the samples were autoclaved for 5 minutes at 15 lb. after adjusting the pH to from 6.8 to 7.0 to avoid alkaline destruction of vitamin B_{12}. Finally, the samples were filtered and diluted to proper levels. Vitamin B_{12} was determined by a modification of the method of Skeggs et al. ('48). An amino acid mixture was used instead of the acid-hydrolyzed casein; ascorbic acid and ammonium chloride were added to the media and the sodium acetate was increased to a level of 3.2 gm per 100 ml of media. The organism used was *L. leichmannii* (American Type Culture Collection 4797). The unknown factor was determined using *Leuconostoc citrovorum* 8081 as the test organism and following the method of Sauberlich and Baumann ('48). A slight modification was made, in that acid-hydrolyzed casein was used instead of the amino acid mixture. Acid production was measured at 72 hours.

Two experiments were conducted in the course of these studies. In the first experiment diets A-1 to A-6 were used. Four groups of 4 pullets each received the sucrose-basal diet supplemented with 0, 2.5, 5 and 10 gm of APF concentrate R-1 per kilogram of ration, respectively. Two other groups received the starch-basal diet (A-5) unsupplemented, and with 2.5 gm of the same APF concentrate per kilogram of ration (A-6). Another group of 4 pullets received a practical all-mash diet as a positive control.

In the second experiment three different concentrates were used. One was APF concentrate 1 and the other two were APF concentrates R-1 and 197 B. Five groups of 4 hens each were placed on diets A-1, A-11, A-12, A-13 and A-14, respectively. The first group received the basal diet (A-1) low

in vitamin B_{12}. Two groups received this basal diet supplemented with 5 gm (diet A-11) and 10 gm (diet A-12) per kilogram, respectively, of concentrate no. 1. Two other groups received 15 gm of APF concentrate R-1 (diet A-13) and 10 gm of APF concentrate 197 B (diet A-14) per kilogram, respectively. A 6th group of 4 hens was placed on a practical all-mash diet.

Hemoglobin and blood glucose determinations were run at the end of the second experiment on the hens of the groups fed the basal diet (A-1), the diet supplemented with 10 gm of concentrate 1 per kilogram (A-12), and the practical all-mash diet. Hemoglobin determinations were made by the direct photometric method (Hawk et al., '47) and blood glucose was determined colorimetrically by the method of Somogyi ('45).

RESULTS AND DISCUSSION

First experiment

Pullets fed diet A-1 had a lower percentage egg production than did any group fed sucrose supplemented with different levels of R-1 (diets A-2, A-3, A-4). The production of the latter groups for the 9-week period ranged from 62 to 66%, while that of the unsupplemented group was 48%. The percentage decreased to 29% in the group fed diet A-1 during the final week of the experiment.

The percentage egg production for the 9-week period of pullets fed starch as a source of carbohydrate (diets A-5 and A-6) was about the same as that of those fed the practical diet and slightly higher than that of those fed sucrose (diets A-1, A-2, A-3, A-4).

The per cent hatchability of the groups fed the sucrose diets is shown in figure 1. In the group fed the sucrose diet with no supplement (diet A-1) the hatchability dropped to zero at the end of the third week. During the 4th week live chicks from two hens were obtained, but after this time hatchability dropped to zero again and remained at zero

through the 9th week. Hatchability in the other groups fed sucrose (diets A-2, A-3, A-4) was better than that of the group fed the deficient diet but not as good as that of the group fed the practical diet. There was a correlation between the amount of concentrate fed and hatchability, although there was a tendency for the hatchability in all of the supplemented groups to decrease toward the end of the experiment. No live chicks were obtained from pullets fed sucrose with 2.5 gm of concentrate (diet A-2) during the 9th week.

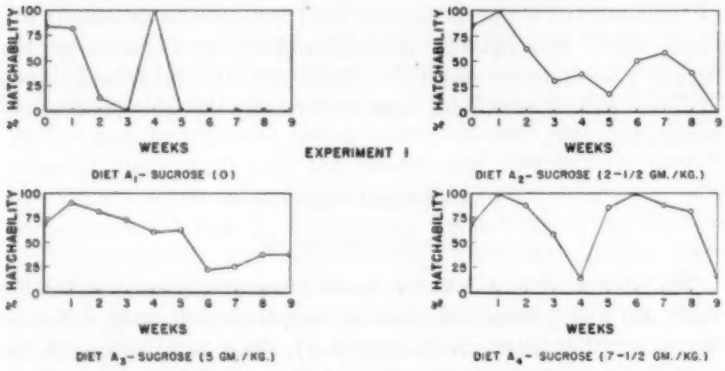
% HATCHABILITY
WEEKS
EXPERIMENT I
DIET A1 - SUCROSE (0)
DIET A2 - SUCROSE (2-1/2 GM./KG.)
DIET A3 - SUCROSE (5 GM./KG.)
DIET A4 - SUCROSE (7-1/2 GM./KG.)

Fig. 1 Effect on hatchability of supplementing sucrose-low vitamin B_{12} diets with different amounts of APF concentrate R-1.

The hatchability of eggs from pullets fed the starch diets was better than that of the equivalent groups fed the sucrose diets, as can be seen in figure 2. The hatchability of the eggs from the group fed starch and no APF concentrate (diet A-5) did not drop to zero until the 9th week, although it remained below 50% after the third week. The per cent hatchability of eggs from pullets fed diet A-5 (starch) is comparable to that of those fed the sucrose diet supplemented with 2.5 gm of the APF concentrate (diet A-2). The hatchability of eggs from pullets fed the starch diet supplemented with 2.5 gm of APF concentrate (diet A-6) was over 50% except for the 5th and 9th weeks, and is comparable to that

of those fed sucrose and 10 gm of APF concentrate (diet A-4). In this experiment the per cent hatchability of eggs from birds fed starch was higher than that of comparable groups fed sucrose (figs. 1 and 2). This might have been due to the synthesis of vitamin B_{12} and unidentified factors in the gastrointestinal tract of the pullets fed starch. If this is the case, when no supplement was fed with the starch diet the limiting factor may have been vitamin B_{12}, but when 2.5 gm of concentrate were fed with this diet another factor

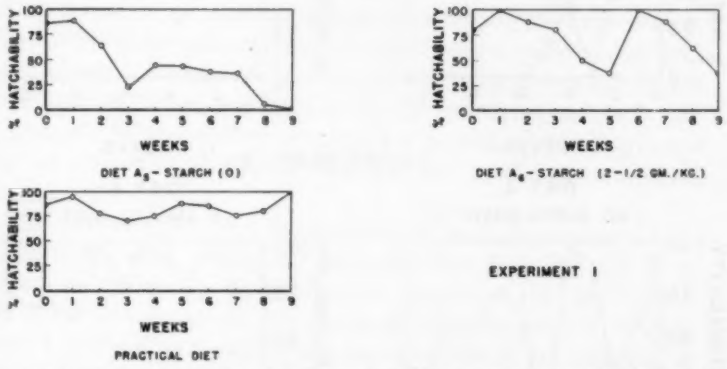

Fig. 2 Effect on hatchability of supplementing a starch-low vitamin B_{12} diet with 2.5 gm of APF concentrate R-1.

may then have become the limiting factor, and the starch diet may have promoted an increased synthesis of this factor as well as of vitamin B_{12} and produced a further response in hatchability.

The group fed the practical diet showed the highest percentage hatchability in the test. This fact seems to indicate either that the amounts of supplements used were too low and did not meet the requirements of the mature fowl for vitamin B_{12} or that an unknown factor is required, in addition to vitamin B_{12}, for normal hatchability in the mature fowl.

Second experiment

The egg production of the pullets fed the basal diet A-1 in the second experiment was again lower than that of the hens fed any of the supplemented diets or the practical diet. When the entire experimental period is considered, the per-

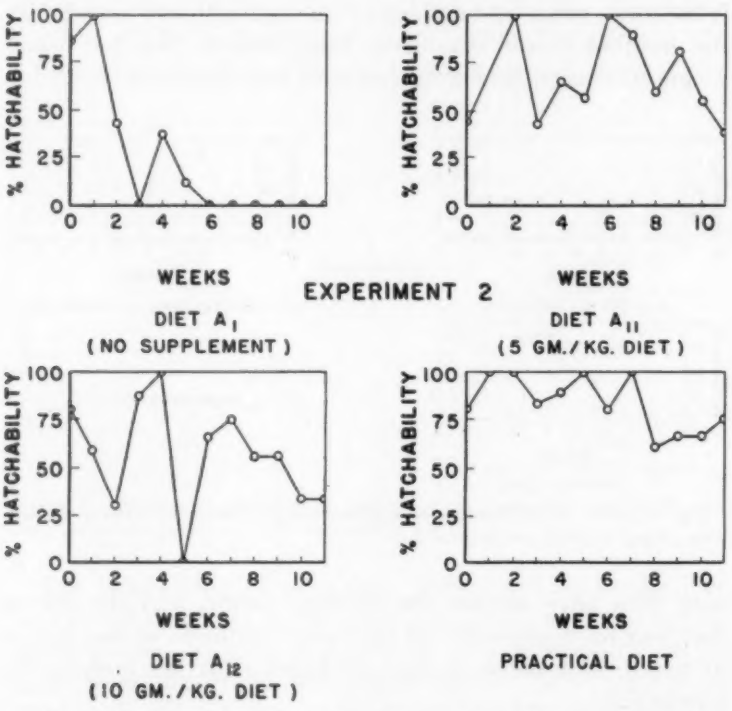

Fig. 3 Per cent hatchability of eggs from pullets fed concentrate 1.

centage egg production of the groups fed 5 gm (diet A-11) and 10 gm (diet A-12) of concentrate 1 was 74 and 62, respectively, and for the two groups fed concentrates R-1 (diet A-13) and 197 B (diet A-14) the percentage egg production was 68 and 66, respectively. The egg production of the group

fed the practical diet over the whole experimental period was 72%.

As in the previous experiment, we may conclude that a deficiency of vitamin B_{12} decreases egg production.

The effect on per cent hatchability of supplementing a low-vitamin B_{12} diet with different amounts of concentrate 1 is shown in figure 3. The hatchability of eggs from pullets fed the basal diet (A-1) dropped to zero during the third week, increased to 43% during the 4th week, and dropped again to zero in the 6th week. The hatchability of eggs from this group remained at zero throughout the rest of the experiment. The percentage hatchability of eggs from pullets fed diet A-11 (5 gm concentrate 1 per kilogram) was somewhat better than that of those fed diet A-12 (10 gm concentrate 1 per kilogram). Hatchability of eggs from both of these groups (fig. 3) decreased decidedly near the end of the experimental period.

The effect on per cent hatchability of supplementing a low-vitamin B_{12} diet with APF concentrates R-1 and 197 B is shown in figure 4. In these two instances the percentage hatchability was better than that of the groups fed concentrate 1 and did not exhibit the same tendency to decrease at the end of the experimental period which was so apparent with birds fed diets A-11 and A-12 (figs. 3 and 4). These data would seem to indicate that concentrates R-1 and 197 B may have contained another factor in addition to vitamin B_{12} which is necessary for embryonic development. Stokstad et al. ('49) reported that such concentrates contain a factor which increased chick growth over that observed when vitamin B_{12} (crystalline) was injected.

The levels of APF concentrates used in this experiment were higher than in the previous experiment and presumably high enough to meet the requirements of the hen for vitamin B_{12}. The groups fed concentrate 1 were receiving approximately 18 and 36 μg of vitamin B_{12} in the supplement per kilogram of ration, as shown by microbiological assay with *L. leichmannii* (American Type Culture Collection 4797).

However, the possibility that a certain amount of vitamin B_{12} was not available for this organism must be considered, since this concentrate has been reported to contain up to 2 mg per pound (4.3 μg/gm) and, on the basis of this figure, our rations would contain 21.5 and 43 μg of added vitamin B_{12}

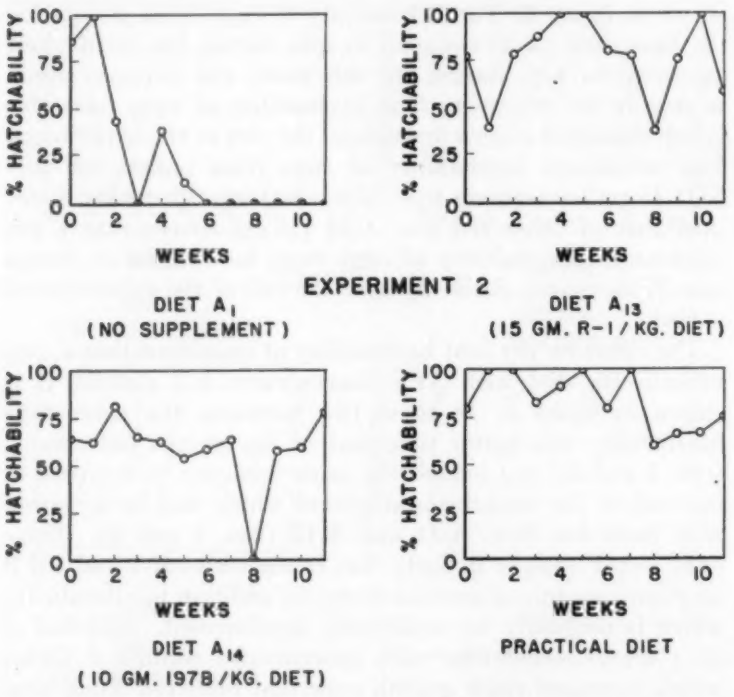

Fig. 4 Per cent hatchability of eggs from pullets fed concentrates R-1 and 197 B.

per kilogram of ration. Recently Ott, Rickes, and Wood ('48) found that 6 μg of vitamin B_{12} per kilogram of diet stimulated the growth of chicks as much or more than dietary supplements of crude sources of this factor. In a later study Ott ('49) used depleted chicks from hens fed an all vegetable

protein ration and stated that the minimum vitamin B_{12} requirement of the chick for maximum early growth was 27 μg per kilogram of diet. Nichol, Dietrich, Cravens and Elvehjem ('49) have reported that for chicks vitamin B_{12} at the level of 15 μg per kilogram of ration is as good as 3% of fish solubles in the diet or as Reticulogen given intravenously at a dosage of 0.5 U.S.P. units per bird per day.

The diets supplemented with concentrates R-1 and 197 B gave a lower *L. leichmannii* assay value than did those containing concentrate 1. This may have been due to the presence of a bound form of vitamin B_{12} in the former materials. The fact that the hatchability with both concentrates R-1 and 197 B was better than that of the groups fed concentrate 1 seems to indicate the presence of other factors in the former concentrates which are presumably required by the hen in addition to vitamin B_{12}. The possibility also exists that a certain amount of vitamin B_{12} was not available for detection with *L. leichmannii* in these two concentrates.

Determination of vitamin B_{12} and the L. citrovorum factor in the eggs

Vitamin B_{12} assays were run on pooled weekly samples of egg yolks from each group of hens in the second experiment. The ranges used were from 5 to 25 mg of egg yolk (fresh weight basis) per tube. It is obvious from the data in figure 5 that the apparent vitamin B_{12} content of egg yolk from hens fed diet A-12 was higher than that of the other groups although, as discussed above, the hatchability of this group was lower than that of those fed concentrates R-1 and 197 B or the practical diet. This indicates further that vitamin B_{12} may not have been the limiting factor associated with the failure of embryonic development in those groups fed concentrate 1 (diets A-11 and A-12). The apparent vitamin B_{12} content of eggs from pullets fed concentrate R-1 (diet A-13) was almost identical with that of the group fed diet A-11 (fig. 5), and the vitamin B_{12} content of the eggs from

the group fed supplement 197 B (diet A-14) was about the same as that of eggs from pullets fed the practical all-mash diet (fig. 5).

There was no correlation between the *Leuconostoc citrovorum* factor content of egg yolks and the amount of vitamin B_{12} concentrate fed. The eggs from hens fed the practical diet contained no more of this factor than did those from the other groups in the second experiment.

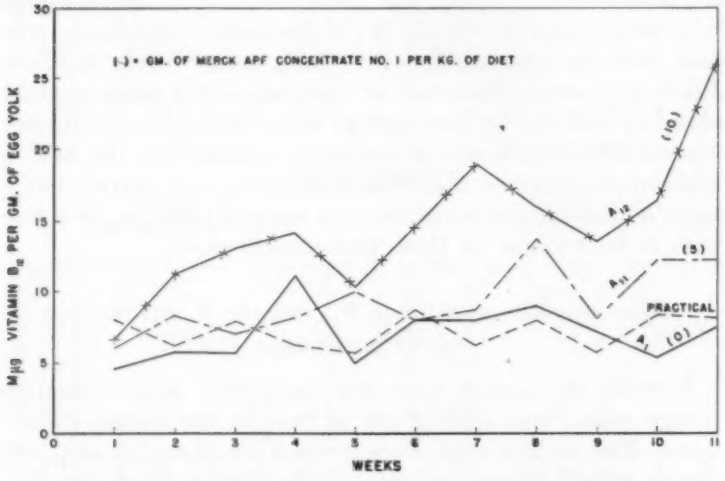

Fig. 5 Effect of feeding different amounts of APF concentrate 1 on the apparent vitamin B_{12} content of egg yolk.

Hemoglobin and blood glucose

Hemoglobin and blood glucose were determined on birds from the groups fed the basal diet (A-1), diet A-12 (10 gm concentrate 1 per kilogram) and the practical diet at the end of the second experiment. The average hemoglobin values were 8.0, 9.0 and 8.4%, respectively, which are about the same as those reported by Dukes ('47) for the hemoglobin level of the hen.

The average blood glucose values for the groups fed the basal and the practical diets averaged 149.3 mg% and 148.5 mg%, respectively. Addition of the vitamin B_{12} concentrate (diet A-4) increased the blood glucose value to 173.3 mg%. The above values are within the range of blood sugar values reported by Dukes ('47). It should be emphasized that the blood sugar values reported above were those of birds that had been without feed for 24 hours. In a recent paper Williams, Nichol and Elvehjem ('49) reported that pteroylglutamic acid tended to decrease the blood sugar level of the chick, whereas vitamin B_{12} was without effect on this component.

The results of these experiments indicate that the addition of crude sources of vitamin B_{12} to a purified diet for hens does not support normal hatchability of the eggs. The hatchability decreased toward the end of the experiment even though the hens received amounts of vitamin B_{12} which presumably were high enough to meet the requirements of the laying hen. The data indicate that an unidentified factor or factors may be required by this species for normal hatchability, and that the depletion of this factor does not take place until several weeks after the depletion of vitamin B_{12}.

SUMMARY

1. When a sucrose-soybean protein diet, low in vitamin B_{12}, was fed to a group of hens, egg production decreased. Hatchability of eggs from hens fed this low vitamin B_{12} diet decreased to zero in from 3 to 6 weeks.

2. The addition of APF concentrates improved the egg production and hatchability of hens fed the purified diet but failed to produce normal hatchability as compared to a group fed a practical all-mash diet.

3. When a starch-soybean protein diet was used, egg production and hatchability were improved over those observed with comparable groups fed sucrose as a source of carbo-

hydrate, indicating that starch promoted the intestinal synthesis of vitamin B_{12} and possibly some unknown factor required for embryonic development in the fowl.

4. The hatchability of eggs from hens receiving the purified diets showed a tendency to decrease about the 9th or 10th experimental week, even though adequate amounts of vitamin B_{12} were fed. This indicates that a depletion of some unknown factor or factors may have taken place after the initial depletion of vitamin B_{12}.

ACKNOWLEDGMENTS

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AMINO ACID IMBALANCE AND THE GROWTH REQUIREMENTS FOR LYSINE AND METHIONINE

C. R. GRAU AND M. KAMEI
Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley

TWO FIGURES

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As our knowledge of the dietary amino acid requirements of various animals increases, the complexity of the interrelationships among amino acids and other nutrients becomes more apparent. The work of Womack and Rose ('47) and of Rose, Oesterling and Womack ('48) with rats, of Maddy and Elvehjem ('49) with mice, and of Almquist and Grau ('44), Grau ('47) and McKittrick ('47) with chicks was done to determine the optimum proportions and amounts of the various amino acids needed in the diets of these animals for satisfactory growth. Brickson, Henderson, Solhjell and Elvehjem ('48), among others, have studied amino acid balance in the nutrition of microorganisms.

Geiger ('48) has reviewed briefly the work with rats which shows that all essential amino acids must be supplied simultaneously if the animal is to use them satisfactorily. Similar work with the chick has been reported by Almquist ('47).

It has been observed that the addition to the diet of excess DL-methionine adversely affects the growth rate of chicks (McKittrick, '47) and of rats (Kade and Sheperd, '48). McKittrick reported that glycocyamine or serine counteracts this growth-depressing effect. Phenylalanine levels above the optimum were found to have a growth-depressing effect on

chicks when their diet contained 2.2% tyrosine, but not when it contained 1.1% tyrosine (Grau, '47).

The addition of gelatin, zein, or certain amino acids to rat or chick diets supplying marginal levels of tryptophan and nicotinic acid has been found to increase the nicotinic acid requirements of these animals (Briggs, '45; Krehl, Sarma and Elvehjem, '46; Groschke, Anderson and Briggs, '48). It has been suggested by Briggs, Groschke and Lillie ('46) that these effects may explain the results obtained by Hier, Graham, and Klein ('44), who observed that even when it is supplemented with all reported essential amino acids, gelatin does not allow optimum growth, and that among rats poorer growth is obtained with a mixture of gelatin and amino acids than with the amino acids alone.

High protein levels have been found to increase the requirements of chicks (Rubin and Bird, '47) and rats (Zucker and Zucker, '48) for the "animal protein factor."

It has been reported previously that the lysine requirement of the young chick increases as the protein level is increased, even though increasing the protein level to above 20% of the diet fails to increase the maximum attainable growth rate (Grau, '48). In the present paper we are reporting our studies of: (1) the chick's lysine requirement, as it is affected by adding zein or an amino acid mixture to the diet; and (2) the effect of the protein level of the diet on the methionine requirement of chicks. Attempts have been made to determine whether particular amino acids are responsible for the effects observed.

METHODS

The methods used in these studies were those reported previously (Grau, '48), namely: White Leghorn chicks were reared on a commercial-type mash (stock diet) for the first two weeks after hatching, then were segregated on the basis of weight into groups of from 7 to 10 chicks and given the experimental diets. The birds were housed in electrically heated battery brooders with wire floors; feed and water were

supplied ad libitum. The chicks were weighed every other day during an 8-day period. The experimental diets contained a basal mixture of salts and vitamins,[1] appropriate protein sources and free amino acids, as noted below, and glucose (cerelose) to make up to 100 gm. The growth rates were calculated by multiplying the average gain per chick per day by 100 and dividing by the average weight during the experiment, which gives the per cent gain per day (Grau and Almquist, '43).

Lysine

Sesame seed oil meal, a readily available feedstuff and a good source of all the essential amino acids except lysine (Grau and Almquist, '44), was used as the protein source in the lysine studies. To take into account the possibility that sesame seed oil meal contains some material peculiar to it which affects the lysine requirement, the level of sesame seed protein was kept constant while the zein and lysine levels were varied. Also, it was desirable to use diets containing zein as well as sesame protein because it is difficult to formulate a diet with a protein level above 35% using only sesame seed oil meal, since this feedstuff contains approximately 44% crude protein ($N \times 6.25$).

Experiment 1 (table 1). The basal level of supplemental lysine was 0.3%, which is sufficient to allow optimum growth (6.0% daily gain) at the 20% level of sesame seed protein.

[1] The basal mixture of salts and vitamins consisted, in grams, of calcium gluconate 8, tricalcium phosphate 2, sodium dihydrogen phosphate 1, potassium chloride 0.6, sodium chloride 0.5, choline chloride 0.2 (provided by Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Dr. T. H. Jukes), crude soybean oil 5, a 15% concentrate of natural mixed tocopherols 0.05 (Distillation Products, Inc.), fortified sardine oil (3,000 A–400 D per gram) 0.25 gm per 100 gm diet, and (in milligrams) of thiamine 1, riboflavin 1, pyridoxine 1, nicotinic acid 5, calcium (*d*) pantothenate 3, 2-methyl-1,4-naphthohydroquinone diacetate 1, biotin 0.1, pteroylglutamic acid (folic acid; provided by Lederle Laboratories) 1, manganese sulfate 30, sodium silicate ($9H_2O$) 200, magnesium sulfate ($7H_2O$) 600, aluminum sulfate ($18H_2O$) 100, ferric citrate 74, copper sulfate 5, zinc sulfate ($7H_2O$) 5, potassium iodide 1, cobalt acetate ($4H_2O$) 2 mg per 100 gm diet.

A higher lysine level (0.5%) did not promote significantly better growth. These observations confirm results reported previously (Grau, '48). When 10% zein replaced 10% glucose, the growth rate was reduced to 5.3% per day; 20% zein decreased the growth rate to 3.4% per day. Contrary to our expectations, adding lysine when the level of supplementary zein was 10% did not increase the growth rate significantly. At the 20% level of zein, however, the addition of 0.5% lysine produced a marked effect.

TABLE 1

Effects of addition of zein or an amino acid mixture on the lysine requirement of chicks. The basal diet contained 20% protein from sesame seed oil meal. The supplements replaced their respective weights of glucose

EXPERIMENT NO.	ZEIN	DL-TRYPTOPHAN	AMINO ACID MIXTURE [1]	GROWTH RATES (% GAIN PER DAY) AT SUPPLEMENTAL LYSINE [2] LEVELS OF					
				0.3%	0.4%	0.5%	0.7%	0.9%	1.1%
	%	%							
	0	0	—	6.0		6.2			
	0	0	—			6.6			
1	10	0	—	5.3		5.5			
	20	0	—	3.4		5.0			
	0	0	+	6.0		6.1			
2	0	0.2	—	6.6	6.8	6.8			
	20	0.2	—	3.6	4.5	5.0			
3	0	0.2	—	6.2					
	20	0.2	—	3.2		5.2	6.2	6.7	6.5

[1] This mixture added to the diet the following percentages of amino acids: L-arginine monohydrochloride 0.5, DL-methionine 0.5, and DL-phenylalanine 0.5.

[2] Lysine supplements were in the form of L-lysine monohydrochloride monohydrate, but are expressed as free lysine.

Sesame seed protein contains comparatively high levels of arginine, methionine, and phenylalanine (Grau and Almquist, '44), and in order to determine whether or not these amino acids were responsible for the effect of protein observed previously (Grau, '48), we added a mixture of these amino acids to diets containing both levels of lysine. Optimum growth was observed in the chicks fed both diets; hence we may safely reject the hypothesis that comparatively small

increments of these particular amino acids were responsible for the protein effect on the lysine requirement.

Experiment 2. In this experiment, only the 20% level of supplemental zein was used, while three levels of supplemental lysine were fed. The resulting growth rates (table 1) clearly indicate that the addition of zein increased the requirement for lysine; even the highest level of lysine fed (0.5%) was insufficient to promote optimum growth. In this and the third experiment, 0.2% DL-tryptophan was added to all diets in order to compensate for a possible tryptophan deficiency caused by the addition of zein. The resulting growth rates apparently were not affected by the tryptophan supplement (compare growth rates in experiments 2 and 3 with those in experiment 1).

Experiment 3. In this experiment, graded levels of supplemental lysine were added up to 1.1%. It was found that the lysine requirement existing when 20% zein had been added to the diet was satisfied by 0.7% supplemental lysine. The addition of larger amounts of lysine did not appreciably increase the growth rate.

The agreement of results among the three experiments is generally satisfactory. With the diets containing 20% zein, for instance, the growth rates were 3.4%, 3.6%, and 3.2% at the 0.3% supplemental lysine level and 5.0%, 5.0%, and 5.2% at the 0.5% supplemental lysine level.

On the basis of these results, it appears that the supplemental lysine requirement under our experimental conditions was approximately 0.7%. Since the basal diet contained 0.56% lysine (Grau, '48), the total lysine requirement with a diet containing 20% zein is approximately 1.36%.

Methionine

Almquist ('47), reviewing the available data on the methionine and cystine requirements of the chick, concluded that when the diet contains 20% to 25% crude protein, 0.9% methionine, or 0.5% methionine plus 0.4% cystine, are required.

We have investigated the requirement for methionine plus cystine at several levels of protein. The amino acid source used was isolated soybean protein,[2] which contained 82.6% crude protein (N × 6.25), 1.5% methionine, and 0.17% cystine. The methionine content was determined by the methods of McCarthy and Sullivan ('41) after pepsin hydrolysis by the

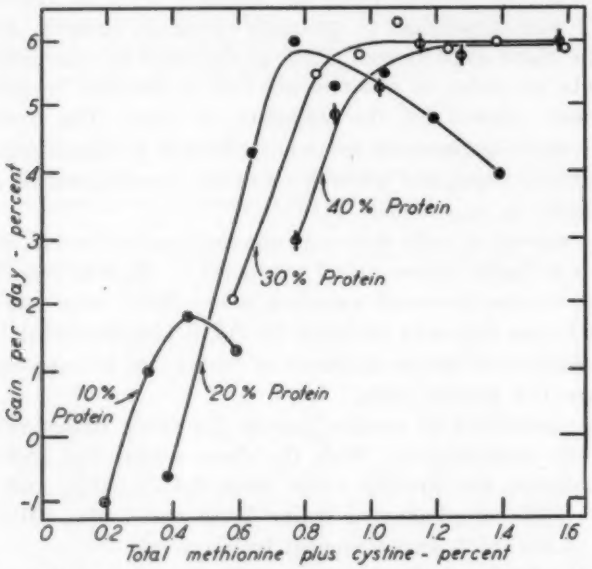

Fig. 1 The effect of protein level on the chick requirement for methionine plus cystine. Each point represents a group of 8 chicks. See also text footnote 3, p. 95.

Grau and Almquist ('45) method; cystine was determined by the colorimetric method of Rossouw and Wilken-Jorden ('35) as modified by Sullivan and Hess ('37).

The supplement used in all diets was DL-methionine, which served the chicks as a source of both methionine and cystine. The results of the first experiment are presented in figure 1. When 10% protein was fed, the supplements of methionine

[2] "Alpha" protein from the Glidden Co., Chicago, Ill.

increased the rate of growth to a maximum of 2% per day; to attain this level, 0.45% methionine plus cystine was needed.[3] At the 20% protein level, maximum growth rates were obtained with 0.78% methionine plus cystine. Above this methionine level the growth rate was found to decline; these observations are in agreement with the results of McKittrick ('47).

At the 30% protein level, the requirement for methionine plus cystine is definitely higher than at the 20% protein level, although the maximum growth rate is no higher. This increased requirement is qualitatively parallel to the effect of the protein level on the lysine requirement (Grau, '48). At the 40% protein level, the methionine plus cystine requirement is greater than at the 30% level.

An interesting and important difference among the growth results at the 20%, 30% and 40% protein levels is observable at the higher levels of methionine, where the higher protein levels protect against the growth-depressing effect of excess amounts of this amino acid.

Because growth rates for the 20% and 30% protein levels were found to differ markedly at the higher methionine levels, an experiment was performed to determine the effects of supplementing with graded levels of methionine a 20% protein diet containing 0.4% added L-cystine. The resulting poor growth at high methionine levels (table 2) confirms the preceding observations.

Another aim of this study was to estimate the cystine requirement of growing chicks receiving a basal diet containing 0.5% methionine. The amino acid sources used were commercial casein and isolated soybean protein (11.5% of each), arginine monohydrochloride 0.2%, and glycine 0.5%. The choline chloride content was 0.3%. Calculations based upon published analyses and those performed in this laboratory indicated that the basal diet contained 0.5% methionine,

[3] In figure 1 no correction is made for the differences in molecular weight between methionine and cystine because the cystine content of all diets is low and varies directly with the protein level.

0.07% cystine, and 20% crude protein (N × 6.25). The addition of several levels of cystine to the diets fed groups of 10 chicks each produced the growth rates presented in figure 2. From these data it appears that a supplement of slightly more than 0.2% L-cystine was required, under our conditions, for satisfactory growth. Since the basal diet contained 0.07% cystine, the total cystine requirement is satisfied by slightly more than 0.27% L-cystine when 0.5% methionine is present.

From the data given in figures 1 and 2, estimates can be made of the methionine and cystine requirements of the chick. At the 20% protein level, the minimum requirement for methionine plus cystine is approximately 0.8% of the diet. From the data shown in figure 2 the cystine requirement is estimated to be approximately 0.3% when 0.5% methionine is present. Since this cystine is not used for methionine replacement (Almquist, '47), we are reasonably safe in estimating the methionine requirement to be 0.5% of the diet. This last figure agrees very well with the previously published estimate of Almquist ('47), but our estimated cystine requirement is lower than that suggested by him.

TABLE 2

Effect of supplementing a 20% protein diet with graded levels of DL-*methionine. The basal diet contained 0.35% methionine and 0.44% cystine*

DL-METHIONINE SUPPLEMENT	GAIN PER DAY
%	%
0	6.2
0.1	7.0
0.2	6.3
0.3	6.6
0.4	5.9
0.5	5.8
0.8	4.9

An attempt was made to simulate a high protein level by adding single amino acids to a diet containing 20% crude protein plus an adequate but not excessive level of methionine. If glutamic acid, glycine, leucine, or phenylalanine additions

produced the same effect on the methionine requirements as are observed at high protein levels, then adding graded levels of methionine and one of these amino acids would be expected to show the following results: At a methionine level barely adequate for optimum growth, the addition of 10% of one of the amino acids mentioned would lower the growth rate. Adding methionine to this diet should then restore the higher growth rate. To test this hypothesis, the experiment the results of which are tabulated in table 3 was conducted. Two

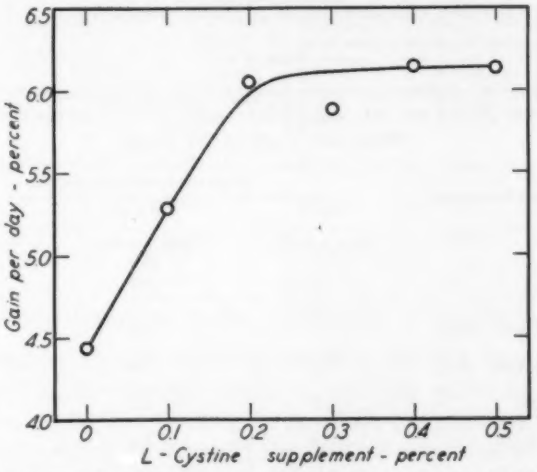

Fig. 2 The growth response to cystine added to a diet calculated to contain 0.5% methionine and 0.3% choline chloride.

groups were fed the basal methionine level in order to provide a double control. The addition of more methionine had a slight depressive effect on their growth rates.

When 10% L-glutamic acid replaced 10% glucose, a slight depressive effect on growth was noted. Twenty per cent L-glutamic acid produced a significant growth-depressing effect. Ten per cent L-leucine was more harmful than an equal level of glutamic acid; glycine and DL-phenylalanine were even greater growth depressants. Adding methionine to diets containing supplements of these amino acids had no effect

on the observed growth rates, thus failing to confirm the hypothesis.

It is, of course, possible that the addition of these amino acids increased the requirement for some other nutrient. It is known, for example, that glycine additions increase the nicotinic acid requirements of chicks (Groschke and Briggs, '46); however, on the basis of published observations, we concluded that the supplement of 5 mg nicotinic acid per 100 gm diet would satisfy even the increased requirement for this vitamin created by a glycine addition.

TABLE 3

The effects on the methionine requirement of chicks of adding amino acids to a 20% protein diet. The supplements replaced glucose in the diet. (There were 8 chicks per group)

AMINO ACID SUPPLEMENT	DL-METHIONINE SUPPLEMENT		
	0.4%	0.6%	0.8%
	gain per day, %	*gain per day, %*	*gain per day, %*
None	6.0	5.6	5.6
None	6.4		
L-Glutamic acid, 10%	5.7	4.5	5.4
L-Glutamic acid, 20%	2.8	1.9	2.5
Glycine, 10%	1.0	1.2	
L-Leucine, 10%	3.5	3.0	
DL-Phenylalanine, 10%	—1.2	—1.4	

DISCUSSION

From the data presented in this paper and those published previously (Grau, '48) table 4 has been prepared, to facilitate comparison of the lysine and methionine plus cystine requirements at several protein levels, expressed as percentages of the diet and of the protein. Rates of growth were suboptimum at protein levels below 20%.

It is apparent from the data of table 4 that as the protein level is increased the lysine or methionine plus cystine requirements also increase but at a slower rate. Thus, a protein

that contains 3.8% lysine will not satisfy the lysine requirement if it is fed at a level providing 20% protein in the diet but will be satisfactory at a dietary protein level of 30%.

These observations are not in agreement with those of Almquist [4] ('49), which indicate that the rate of increase of the methionine requirement is the same as that of the protein level.

TABLE 4

Comparisons of lysine and methionine plus cystine requirements at several levels of dietary protein

PROTEIN LEVEL	AMINO ACID LEVEL REQUIRED FOR MAXIMUM GROWTH			
	Lysine		Methionine plus cystine	
%	*% of diet*	*% of protein*	*% of diet*	*% of protein*
5 [1]	0.25	5.0	. . .	. .
10 [1]	0.48	4.8	0.45	4.5
20	0.85	4.25	0.78	3.9
30	1.15	3.8	1.0	3.3
40	1.36	3.4	1.2	3.0

[1] The best growth attained with these protein levels was suboptimum.

A possible, though unlikely, explanation for this discrepancy is that the cystine requirement for optimum growth is unaffected by the protein level, while the methionine level is markedly affected. Thus, if the cystine requirement (0.3%) were subtracted from the methionine plus cystine requirement at all protein levels, it might be found that the methionine requirement increases at the same rate as the protein level. (It should be added here that no direct evidence is yet available concerning the cystine requirement and its relation to the protein level.)

SUMMARY

As the protein level of the chick's diet is increased, the lysine and methionine plus cystine requirements also increase but at a slower rate. Thus, a protein which is slightly de-

[4] We are indebted to Dr. Almquist for a copy of his paper prior to publication.

ficient in lysine or the sulfur amino acids at the 20% protein level may prove satisfactory when the protein level is increased to 30%.

Attempts were made to simulate the effect of high protein levels by adding free amino acids to diets containing barely adequate amounts of lysine or methionine plus cystine. The growth depressions caused by these amino acid excesses were not correctable by raising the methionine level of the diet.

The growth-depressing effects of DL-methionine levels above the optimum are overcome by high protein levels.

When the diet contains 20% protein, the chick's requirements for methionine and cystine appear to be met by levels of approximately 0.5% and 0.3% of the diet, respectively, or 0.8% methionine alone.

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NUTRITIONAL VALUE OF PLANT MATERIALS

I. GROWTH OF RATS ON PURIFIED RATIONS CONTAINING SOYBEAN PROTEIN[1]

M. O. SCHULTZE

Division of Agricultural Biochemistry, University of Minnesota, St. Paul

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In most purified rations used for studies of the nutritional requirements of the rat and some other species casein, purified to various degrees, has contributed a large share, or all, of the protein. Many recent studies (reviewed by Huff, '49) have indicated that rations composed solely of plant materials and supplemented with synthetic compounds do not support optimum growth and reproduction of rats, mice, chickens and swine unless materials derived from animal or microbiological sources are added.

The so-called "animal protein factor" and vitamin B_{12} (Ott et al., '48; Emerson et al., '49b; Johnson and Neumann, '49; Hogan and Anderson, '49; Stern et al., '49) are considered to be mainly responsible for the superior growth and reproduction of animals maintained on rations containing crude animal products. Soybean meal has been a constituent of many rations with which the effectiveness of the "animal protein factor" and vitamin B_{12} has been demonstrated. The present paper presents data on the growth performance of rats maintained for 4 generations on purified rations in which soybean protein supplemented by DL-methionine furnished the only source of amino acids. Neumann et al. ('48) have

[1] Paper no. 2515, Scientific Journal Series, Minnesota Agricultural Experiment Station.

A preliminary report of some phases of this study was presented at the meetings of the American Institute of Nutrition in April, 1949 (Fed. Proc., *8:* 395).

reported that two-day-old pigs fed a ration containing soybean α-protein and DL-methionine made smaller gains than when the ration was supplemented with the antipernicious anemia preparation Reticulogen [2] or with vitamin B_{12} (Johnson and Neumann, '49). White and Sayers ('42) found that a ration containing 21% of an isolated soybean protein with brewers' yeast as the source of the B vitamins supported the growth of male rats at a rate of only 1 gm per day with an average daily food consumption of 5.5 gm.

EXPERIMENTAL

The composition of the rations shown in table 1 was patterned after that used by Nelson and Evans ('47) except that the fat-soluble vitamins were incorporated into the corn oil. The rations were mixed at frequent intervals and stored at 4°C.

Prior to and concurrent with these experiments the vitamin mixture described was tested with rats on the casein ration C_1 through 5 generations. There was no evidence of progressive deterioration of growth or reproductive performance on the casein ration. A number of rats have survived more than two years on this mixture. It appears, therefore, that any major nutritional defects of the soybean rations must be charged to factors other than the carbohydrate, fat, salts or vitamin mixture used. The addition of biotin to the vitamin mixture (ration S_2) did not significantly increase the 6-week weight gains.

The rations were made up to contain 24% protein. According to the manufacturer of the experimental soybean protein used,[3] "it is prepared from defatted soy grits which have been extracted with dilute acid of pH 4.5 at approximately 130°F. The grits are then extracted with dilute alkali. The extract is screened, neutralized to pH 7.0 with dilute acid and spray dried. The product contains 86% protein and has been subjected to sufficient temperature as to degree and time

[2] Lilly.

[3] The Archer-Daniels-Midland Co., Minneapolis.

to improve its biological value." The amino acid composition of two different batches of the product showed very close correspondence, the means being arginine 6.73%, histidine 1.90%, isoleucine 4.54%, leucine 6.17%, lysine 5.02%, methionine 1.02%, phenylalanine 4.24%, threonine 3.02%, tryptophan 0.97%, and valine 4.34% as determined microbiologically through the courtesy of Dr. K. A. Kuiken, Agricultural and Mechanical College of Texas. For two series of experiments

TABLE 1

Composition of rations

RATION	S_1	S_2	S_3	C_1
Soybean protein (commercial)	274.4	274.4	....	....
Soybean protein (purified)	....	....	234.4	....
Casein (alcohol-extracted)	....	....	....	240.0
DL-Methionine	5.6	5.6	5.6	....
Salts 4 [1]	40.0	40.0	40.0	40.0
Vegetable fat (Crisco)	70.0	70.0	70.0	70.0
Corn oil + vitamin mixture [2]	10.0	10.0	10.0	10.0
Sucrose + vitamin mixture [3]	20.0	20.0	20.0	20.0
Sucrose + biotin mixture [4]	..	10.0	10.0	...
Sucrose	580.0	570.0	610.0	620.0

[1] Salts 4 of Hegsted et al. ('41) modified by decreasing the manganese content to one-tenth of the stated amount, because salts 4 were originally used for work with chickens.

[2] Containing dl-α-tocopherol 5.0 gm; fish liver concentrate 220,000 I.U. vitamin A per gram, 2.275 gm; Delsterol 200,000 A.O.A.C. units vitamin D_3 per gram, 0.375 gm; corn oil to a total of 500 gm.

[3] Thiamine chloride HCl 250 mg; riboflavin 500 mg; pyridoxine hydrochloride 250 mg; calcium pantothenate 2.50 gm; nicotinic acid 1 gm; *i*-inositol 20 gm; choline chloride 50 gm; folic acid 10 mg; para-aminobenzoic acid 500 mg; 2-methyl-1,4-naphthoquinone 250 mg; sucrose to a total of 1,000 gm.

[4] Twenty milligrams *d*-biotin, sucrose to a total of 1,000 gm.

(ration S_3) this product was further purified as follows: one part of soybean protein was moistened with three parts of 95% alcohol;[4] the suspension was then vigorously stirred with 30 parts of distilled water and dissolved by the addition of 5% sodium hydroxide to pH 9.5; the protein was precipitated by adding 5% hydrochloric acid to pH 4.7. After

[4] The alcohol used was 95% ethanol denatured with 9% methanol.

standing overnight in the refrigerator the supernatant was siphoned off. The precipitate was again dissolved at pH 9.5 and reprecipitated at pH 4.7 and 50°C. After standing in the refrigerator overnight the supernatant was again siphoned off and the precipitate stirred with an equal volume of 95% alcohol and filtered. The protein was then suspended in 95% alcohol and extracted for a total of 72 hours with three changes of boiling alcohol and finally dried. The extracts were filtered off with each change of solvent.

Most of the rats used in this study were of a black strain of the "Line 3" maintained in this laboratory for many years. A few rats of a closely related albino strain were also included. Since the performance of the two strains was not different, the data obtained with both strains were pooled. The parent generation of rats used in this study (group 2, table 2) was obtained from stock which had been maintained for two generations on a ration (C_1) containing 24% of alcohol-extracted casein (crude lactic casein extracted in a percolator for 72 hours with 95% alcohol at 55 to 60°C.) in lieu of the soybean protein and DL-methionine. The young were weaned at three weeks of age and thereafter kept in groups of 4 or 5 rats per cage on raised screens. Close obsevation failed to reveal evidence of coprophagy, although this does not exclude the possibility of its occasional occurrence. Weights were taken at weekly intervals and the data reported in table 2 are the weight increments observed during the first 6 weeks of rapid post weaning growth. (The beginning of the 6-week period was the 25th–26th day after birth, to allow for the usual delay in weight gains immediately after weaning at three weeks of age.)

RESULTS AND DISCUSSION

Inasmuch as the strain of rats used in these studies is of relatively small stature, the weight gains of the parent generation listed in table 2 are near the maximum observed for this strain on adequate rations of similar caloric density. With successive generations on ration S_1 there was a tendency for

TABLE 2

Six-week weight gains of rats on purified rations. (Figures in parentheses refer to the number of rats)

GROUP	GENERATION ON SOYBEAN PROTEIN	BASAL RATION	SOURCE OF PROTEIN	SUPPLEMENT	WEIGHT GAINS	
					Males	Females
					gm	gm
1	Parent { Litter mates	C_1	Casein	None	(11) 180.4 ± 18.2 [1]	(19) 129.4 ± 13.0 [1]
2		S_1	Soybean protein	None	(12) 172.1 ± 15.4	(26) 122.7 ± 8.4
3	F_1 [2]	S_1	Soybean protein	None	(31) 175.0 ± 13.6	(32) 111.5 ± 12.5
4	F_2	S_1	Soybean protein	None	(17) 163.4 ± 15.4	(22) 112.0 ± 14.0
5	F_3	S_1	Soybean protein	None	(16) 158.0 ± 19.6	(21) 105.6 ± 16.9
6	F_1 and F_2	S_2	Soybean protein	None		(41) 115.6 ± 14.1
7	F_2 and F_3	S_2	Purified soybean protein	None	(4) 167.7 ± 34.4	(18) 114.4 ± 12.6
8	F_1 [3] { Litter	S_1	Soybean protein	None		(6) 107.8 ± 13.1
9	F_1 [3] { mates	S_1	Soybean protein	2% liver extract 1:20		(8) 116.5 ± 11.9
10	F_1 [3] { Litter	S_1	Soybean protein	None	(19) 162.3 ± 24.0	(19) 119.4 ± 10.2
11	F_1 [3] { mates	S_1	Soybean protein	3% condensed fish solubles	(20) 160.5 ± 20.6	(21) 124.0 ± 9.2

[1] Standard deviation (Snedecor, '46).
[2] First and second litters.
[3] Second to 4th litters.

a decreased growth rate. Comparison of the weight increments of both males and females of the parent generation (group 2) on the soybean ration S_1 with those of the F_3 generation (group 5) reveals a statistically significant decline. Calculation of "t" (Snedecor, '46) gives values of 2.06 for males ("t" at the 5% level of probability is 2.06) and 4.68 for females ("t" at the 1% level of probability is 2.69), respectively. The difference in weight increments between males of the F_1 and F_3 generation was significant at the 1% level of probability, while that between the females was not significant. It is interesting to note that further extensive purification of the commercial soybean protein (ration S_3) did not cause a decline in growth rate, nor did supplementation of the ration with 2% liver extract 1:20 [5] or with 3% condensed fish solubles improve the growth rate significantly.

It is well established that soybean meal is very low in vitamin B_{12} activity (Shorb, '48; Emerson, '49a, b; Hartman et al., '49a; Stern et al., '49) and that it is a poor source of the "animal protein factor" (Zucker and Zucker, '48a; Cary and Hartman, '47). On the other hand, liver extract 1:20 (Bosshardt et al., '49; Zucker and Zucker, '48a) and condensed fish solubles (Zucker and Zucker, '48c; Jaffé and Elvehjem, '47; Mishler et al., '48) are effective supplements for rations low in the "animal protein factor." The isolation of the soybean proteins, and particularly the extensive extraction by boiling alcohol, might be expected to yield a product very deficient in these factors. The continued satisfactory growth of our rats through 4 generations and the failure of liver extract and fish solubles to improve the growth rate significantly are therefore in contrast with the observations of others (Cary and Hartman, '47; Zucker and Zucker, '48a; Stern et al., '49; Emerson, '49a, b; Hartman et al., '49a) who have used soybean meal in their rations.

Administration of crystalline vitamin B_{12} [6] to rats on ration S_3 containing purified soybean protein failed to increase their

[5] Wilson.

[6] Cobione, Merck.

early growth rate, as is shown in table 3. The rats used for this experiment were the offspring of females maintained since weaning on the same ration (group 7, table 2). These animals were kept singly in drawer cages with raised screen bottoms. Half of the matched litter mates were given daily 0.25 μg of vitamin B_{12} by subcutaneous injection. Emerson ('49a) has reported that this quantity of vitamin B_{12} injected subcutaneously into young rats on a ration containing 70% soybean meal produced a marked growth stimulation in a two-week period. In the present experiments no effect of

TABLE 3

Growth and food utilization of rats on a ration containing purified soybean protein (ration S_2)

	UNINJECTED CONTROLS			INJECTED WITH 0.25 μG B_{12} PER DAY		
	Initial weight	14-day weight gain	Gram feed per gram gain	Initial weight	14-day weight gain	Gram feed per gram gain
	gm	*gm*		*gm*	*gm*	
Males (7 pairs)	34.1 ± 5.4 [1]	67.0 ± 9.0	1.89 ± 0.11	33.0 ± 3.7	63.6 ± 7.8	2.02 ± 0.14
Females (10 pairs)	34.5 ± 5.0	61.0 ± 6.9	2.06 ± 0.12	33.9 ± 4.8	62.8 ± 4.9	2.08 ± 0.12

[1] Standard deviation.

vitamin B_{12} could be observed, the differences in weight increments and food utilization being statistically insignificant. In fact, the uninjected control rats made excellent gains for the duration of this experiment.

It is unlikely that a carryover of maternal stores from the parent generation would have appreciable effects in the third filial generation. Furthermore, serious disturbances observed incident to reproduction occurred in the parent as well as in the three filial generations on rations S_1 and S_2, and these could be prevented by the feeding of 2% liver extract 1:20 [7] or 3% fish solubles (Schultze, '49). In this laboratory we have also observed poor growth in the same strain of rats on rations compounded from yellow corn, soy-

[7] See footnote 5, p. 108.

bean meal and alfalfa (Halvorson and Schultze, unpublished data). A partial explanation for these discrepancies may lie in the observations of Hartman et al. ('49b) that rats on rations deficient in the factor X (which may be identical with vitamin B_{12}) can achieve excellent growth if these rations contain "extraordinarily high levels of the 10 B vitamins, methionine, and vitamins C, E and K" and that the feeding of a single dose of feces from these fast-growing rats increased the growth rate of slow-growing rats. Schaefer et al. ('49a, b) observed that the addition of vitamin B_{12} to diets low in choline reduced the incidence of kidney lesions in rats and that dietary choline reduced the requirement of vitamin B_{12} for the growth of chickens. It appears, therefore, that a ration deficient in vitamin B_{12} does not necessarily lead to nutritional failure, provided that other conditions are suitable for the achievement of normal physiological performance. The possibility must also be considered that crude soybean meal in which the antitryptic factor has been destroyed may have a nutritional value which is inferior to that of some of its constituent proteins properly supplemented. There appear to be no comparable experiments on record in which the separated proteins of soybeans have been used as the sole source of protein in purified diets fed several generations of rats.

Zucker and Zucker ('48a, b) have reported a very high post-weaning mortality of rats on an unsupplemented ration containing soybean meal or cottonseed meal as the principal source of protein. In the experiments reported here only two out of over 300 rats which were born to mothers on the soybean protein rations and raised for 6 weeks or longer after weaning died during this period. On rations of yellow corn, soybean meal and alfalfa meal unsupplemented with products of animal or microbiological origin a high incidence of mortality during the 4th and 5th weeks of life also occurs in this laboratory (Halvorson and Schultze, unpublished data). The results reported here indicate that the absence of the "animal protein factor" from an otherwise suitably

supplemented ration does not necessarily lead to the severe growth depression and post-weaning mortality observed in rats on natural grain rations.

The reproductive performance of the rats used in these experiments will be discussed in a later communication.

SUMMARY AND CONCLUSIONS

1. Rats fed purified rations containing 24% protein in the form of a soybean protein preparation and DL-methionine as the only source of amino acids made satisfactory post-weaning weight gains for 4 successive generations.

2. Extensive purification of the soybean protein did not decrease the growth rate of rats consuming this protein as compared to that of animals consuming a less purified preparation.

3. Daily administration of 0.25 μg of crystalline vitamin B_{12} to rats on a ration containing a highly purified soybean protein preparation did not increase the early post-weaning weight gains or the efficiency of food utilization.

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SOME INTERRELATIONSHIPS OF THIAMINE AND FAT IN THE NUTRITION OF THE RAT [1]

M. M. KRIDER [2] AND N. B. GUERRANT

Department of Agricultural and Biological Chemistry, The Pennsylvania State College, State College

THREE FIGURES

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The question of the dependence of body fat synthesis on thiamine intake has been of interest for a number of years. Evans and Lepkovsky ('28) were among the first to suggest that an inverse relationship exists between levels of dietary fat and the thiamine requirement of the rat. The apparent reduction in the thiamine requirement resulting from the addition of fat to a low-fat diet was regarded as a "sparing action." Guha ('31), Evans and Lepkovsky ('32), Evans, Lepkovsky and Murphy ('34), and Guerrant and Dutcher ('34) published data which seemed to support the concept of a "sparing action," although studies by Kemmerer and Steenbock ('33) indicated that the "spared" thiamine did not result in an increase in the concentration of thiamine in body tissues. Arnold and Elvehjem ('39) reported that some thiamine was necessary for the maintenance of fat reserves in the rat even when the diet was high in fat. Other investigators, including Whipple and Church ('36, '37), McHenry ('37a, b, '47), and Longenecker, Gavin and McHenry ('40), have considered thiamine as a limiting factor in fat synthesis.

[1] A preliminary report of this study was presented before the American Institute of Nutrition, Detroit, April 20, 1949.

[2] Now associated with the U. S. Eastern Regional Research Laboratory, Wyndmoor, Penna.

However, the data of Quackenbush, Steenbock and Platz ('42) indicate that thiamine is no more specific for fat synthesis than is pantothenic acid or pyridoxine. A more recent report by Boxer and Stetten ('44) indicates that the limiting factor in fat synthesis in rats subsisting on a low-thiamine diet is the low food intake.

It was apparent from a review of the above reports that the role of thiamine in fat synthesis and the effect of dietary fat on the thiamine requirement have not been adequately elucidated. Although the investigations referred to have, as a group, covered a moderate range of levels of thiamine intake and have involved both fat-free and fat-containing diets, individually, the experiments have been limited in scope. In the studies herein reported a more extensive range in daily thiamine intake and a reasonably wide variation in the fat content of the diets have been employed with a view to obtaining further information on: (1) the effect of thiamine intake on the fat content of the carcasses of rats subsisting on a fat-free diet and on isocaloric diets containing added fat; (2) the effect of the fat content of the diet on thiamine storage in the tissues of rats; and (3) the effect of the above dietary variables on some of the characteristics of the deposited fat.

EXPERIMENTAL

The composition of the basal diets used in the present studies is given in table 1. The diets were prepared at frequent intervals and stored at 5°C. until used.

The thiamine supplement was fed at levels of intake per day of 2, 50, 200, and 1,000 μg, respectively, over the 28-day experimental period. Previous studies had shown that 2 μg of thiamine per day were adequate for reasonably good growth in this particular strain of rat subsisting on a thiamine-free diet. Accordingly, this amount of thiamine was chosen as the minimum supplementary level.

Each rat received, at the beginning of the experimental week, 0.01 ml of a vitamin A and D concentrate, which should

have furnished at least 16 I.U. of vitamin A and 1.56 I.U. of vitamin D per week. While it was recognized that these were not optimum amounts of vitamins A and D, it was considered desirable that rats on the low-fat diet be restricted to a minimum of dietary fat; hence a restricted amount of the fat-soluble vitamin concentrate was used.

TABLE 1

The composition of the basal diets and their relative isocaloric factors

INGREDIENTS	LOW-FAT DIET	10% FAT DIET	20% FAT DIET	SUPPLEMENTARY B VITAMINS [6]	AMOUNT IN MIXTURE	CALCULATED SUPPLEMENTARY INTAKE
	gm	*gm*	*gm*		*gm*	*μg/day*
Sucrose [1]	76	56	40	Riboflavin	0.20	40
Casein [2]	18	18	18	Pyridoxine	0.20	40
Hydrogenated cottonseed oil [3]	0	9	18	Niacin	2.50	500
				Calcium pantothenate	1.25	250
Salt mixture [4]	4	4	4	Inositol	0.50	100
Cellu flour [5]	2	2	2	*p*-Aminobenzoic acid	0.20	40
Isocaloric factor	1.00	0.89	0.80	Choline	...	5,000
				Sucrose (as a carrier)	25.15	
				Total	30.00	

[1] Commercially refined cane sugar.

[2] Extracted to remove B vitamins.

[3] Crisco.

[4] U.S.P. no. 2.

[5] Distributed by Chicago Dietetic Supply House, Inc.

[6] The basal diets were supplemented with the following crystalline B vitamins and with a vitamin A and D concentrate.

Weanling rats, 22 to 28 days of age and weighing 40 to 48 gm, were used as test subjects. The matters of sex, litter and body weight were given due consideration in assigning the animals to experimental groups. The rats were housed individually in all-metal cages which were provided with raised screen mesh floors. Fresh distilled water was supplied ad libitum daily and the rats were weighed at weekly inter-

vals. Food intake for each rat over the 28-day period was restricted to 135 gm of the low-fat diet or its isocaloric equivalent. This quantity of food was apportioned to the test animals over the 28-day test period according to a plan of three feedings each week. The feeding cups were cleaned twice weekly and food spillage was recorded as food refusal. Representative groups of rats subsisting on the above diets received the various intakes of thiamine over the test period. Comparable groups of rats subsisting on the breeding colony ration served as positive controls, while other rats subsisting on the 10% fat diet, without the thiamine supplement, served as negative controls.

The last feeding of the various diets and vitamin supplements was made on the 26th day, the feed cups were removed on the 27th day, and the rats were sacrificed on the 28th day of the test period.

Determination of cardiac thiamine

Measurement of the thiamine content of certain body tissues has been suggested as a means of evaluating the "sparing action" of fat on the thiamine requirement of the rat (Kemmerer and Steenbock, '33). Since the heart usually contains a relatively high concentration of thiamine (Williams, Taylor and Cheldelin, '41), and since this organ is easily removed intact, it was selected as the tissue to be used in the present study.

On sacrificing the rat, the thorax was immediately opened and the heart was removed by means of scissors and tweezers, pressed between layers of filter paper to remove blood and adhering moisture, and weighed. The thiamine content of the heart was determined by the thiochrome method (National Canners Association Research Laboratories, '44) and was expressed as micrograms of thiamine per gram of wet tissue.

Determination of carcass fat

A method was developed for extracting and recovering the carcass fat in a state satisfactory for its characterization.

Briefly, the procedure was as follows: After removal of the heart, the alimentary tract was removed and flushed of residual food. Adhering moisture was reduced by draining and by pressing between filter paper. The emptied organ and the remainder of the carcass were minced by cutting, transferred to a tared watch glass, and weighed. The carcass was dried to constant weight by desiccation over sulfuric acid (Wynn and Haldi, '44) and the weight loss recorded as per cent moisture. The desiccated carcass was further subdivided by crushing and shredding with pliers, transferred quantitatively to an extraction thimble, and extracted for 12 hours in a Soxhlet extractor with petroleum ether (b.p. 39–44°C.). The solvent was removed from the extract at temperatures below 60°C. until further heating caused no significant decrease in the weight of the extract.

Determination of some characteristics of fat

In determining the effects of the dietary variables on the physical properties of the extracted fats, those characteristics used as means of comparison were the index of refraction, the iodine number, and the mean molecular weight. The refractive index was measured by means of an Abbe refractometer at 40°C. The Association of Official Agricultural Chemists (A.O.A.C.) procedure ('46) was employed in determining the iodine number of the fat. The procedure used in determining the mean molecular weight of the fat was an adaptation of the A.O.A.C. method for determination of the saponification number.

RESULTS

The data obtained were summarized and typical summaries are presented in figures 1 to 3, inclusive.

DISCUSSION

a. Effect of the fat content of the diet on the change in weight and on the final dry weight

Rats which had subsisted on isocaloric amounts of three experimental diets showed increases in body weight which

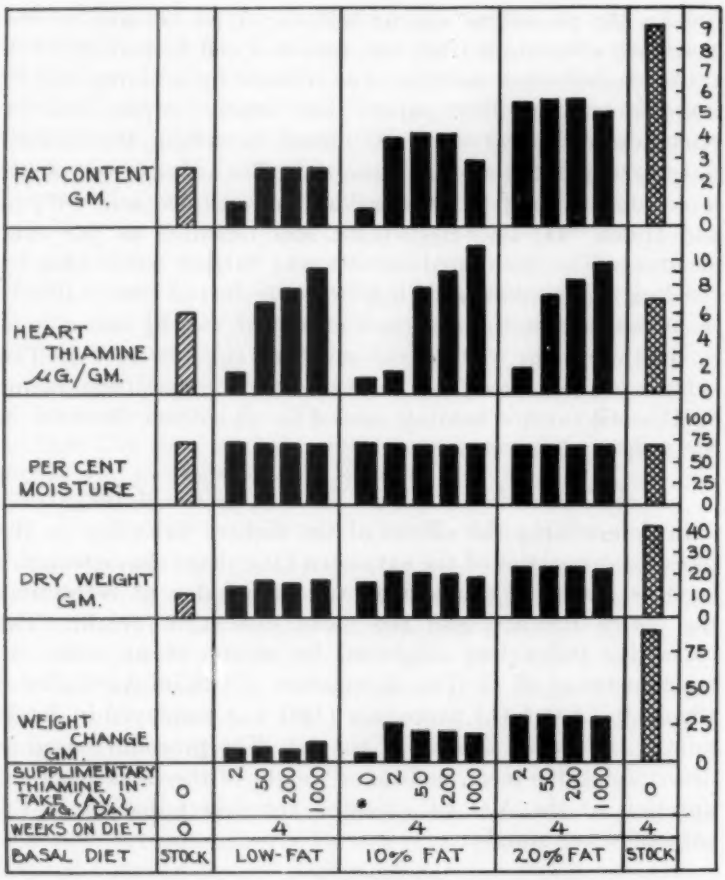

Fig. 1 Effect of the fat content of the diet and of the thiamine intake on growth, fat deposition and thiamine storage.

were proportional to the fat content of the respective diet (fig. 1). This increase in weight was essentially the same with all levels of thiamine intake for those rats which subsisted on the 20% fat diet. However, those rats which received the low-fat and the 10% fat diets showed smaller and some-

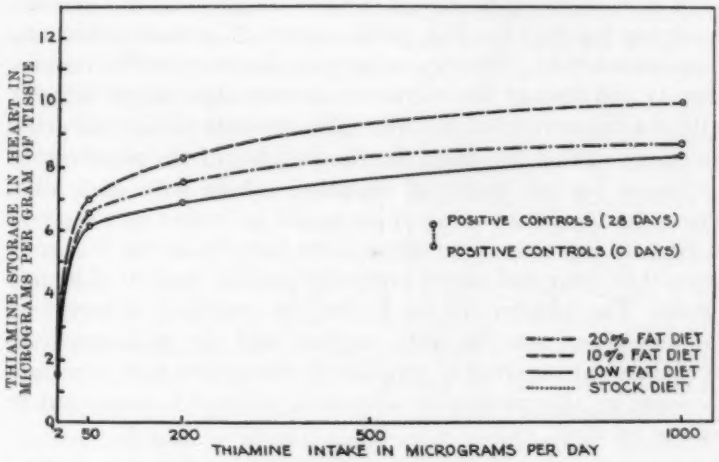

Fig. 2 Relation of the fat content of the diet and of the thiamine intake to the cardiac thiamine.

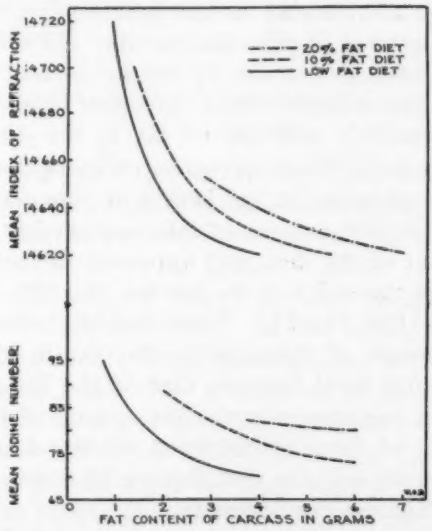

Fig. 3 Relation of the fat content of the carcass to the iodine number and to the index of refraction of the fat.

what less consistent increases in body weight than did animals receiving the 20% fat diet, as the result of increased thiamine supplementation. The dry weight of the unextracted carcass (fig. 1) and that of the extracted carcass also varied directly with the fat content of the diet. The weights of the extracted carcasses varied less than did the weights of the unextracted carcasses for all levels of thiamine intake with each diet. The weights of the extracted carcasses indicate that rats subsisting on any one of the three diets develop about the same amount of bone and tissue regardless of the level of thiamine intake. The addition of fat to the diet resulted in increased fat deposition and the data suggest that the optimum level of dietary fat required to produce the maximum rate of weight increase on this particular allowance of food is somewhat in excess of 20%.

b. Effect of fat content of the diet on cardiac thiamine

The storage of thiamine in the hearts of rats which received the 2 μg level of thiamine per day did not appear to have been affected measurably by the fat content of the diet. However, for rats which received the higher levels of thiamine intake the isocaloric addition of fat to the diet caused a slight increase in the thiamine concentration in the heart. The thiamine concentration in the hearts of rats which had received the 50 to 1,000 μg levels of thiamine per day varied with the fat content of the diet, and appeared to have increased consistently in the order of the low-fat, the 20% fat and the 10% fat diets (figs. 1 and 2). These findings indicate that the maximum storage of thiamine in the rat heart occurs at some dietary fat level between that of the low-fat and the 20% fat diets, perhaps near the fat content of the 10% fat diet. Because of these unsuspected relationships, the data do not necessarily indicate that dietary fat exerts a "sparing action" on thiamine requirements.

Thiamine concentration in the heart varied directly with thiamine intake over the range of levels of 0 to 1,000 μg of

thiamine per day, and the maximum storage of heart thiamine did not appear to have been reached by those rats which received 1,000 μg of thiamine per day (fig. 2).

Schultz, Light, Cracas and Atkin ('39), on analysis of the rat's body (less intestinal contents), concluded that the maximum thiamine concentration was produced by an intake of only 65 μg of thiamine per day. However, for the hearts of 250-gm rats which had subsisted for 4 weeks on diets containing 14% fat these investigators report mean cardiac thiamine storage of 0.68, 2.6, and 7.2 μg of thiamine per gram of heart from animals which had received levels of 0, 15, and 215 μg of thiamine per day, respectively. These values compare favorably with those obtained in the present study. Hence, analyses of tissues from rats which have subsisted on diets supplemented with various levels of thiamine intake for test periods of 4 or more weeks' duration may indicate a greater thiamine storage than is found in tissues from similar test animals slaughtered at the time of thiamine intake-excretion equilibria.

c. Effect of thiamine intake on fat content of the carcass

Thiamine was found to be a limiting factor in fat deposition only over a certain range of thiamine intake, and this particular range of intake was dependent on the fat content of the diet. The critical range of thiamine intake appeared to be between 2 to 50 μg and 0 to 2 μg per day for the low-fat and the 10% fat diets, respectively. In rats which had subsisted on the low-fat diet, fat deposition appeared to be independent of the thiamine intake over a range of levels of from 50 to 1,000 μg of thiamine per day (fig. 1). However, this latter statement does not appear applicable to the data relating to fat deposition in rats which had received the 10% fat or 20% fat diets. The results obtained with these latter diets suggest that there is a certain level of thiamine intake, perhaps at or near 50 μg per day, at which a maximum deposition of fat

occurs in rats subsisting on these fat-containing diets. The data also suggest that fat deposition is suppressed by high thiamine intakes (1,000 μg daily) when the rats are subsisting on diets containing 10% and 20% fat. The reported necessity for supplementing fat-containing diets with some dietary thiamine (Arnold and Elvehjem, '39) has been confirmed by the results obtained with rats which received the 10% fat diet without thiamine supplementation. These negative control animals lost appetite during the second week of the test period and although they survived a 4-week experimental period and their bodies were analyzed, only one rat of the group had consumed the established allowance of food.

d. Effect of dietary variables on characteristics of carcass fat

The index of refraction and the iodine number of the carcass fat were affected by the compostion of the basal diet on which the rat had subsisted during the test period. The ingestion of the respective diets resulted in an increase in the degree of unsaturation of the carcass fat in the order of the low-fat, the 10% fat and the 20% fat diet. The refractive indices and the iodine numbers of the fat from rats which had subsisted on any one of the three diets varied inversely with the percentage of fat in the carcass (fig. 3). Similarly, the molecular weights of these fats varied inversely with the percentage of carcass fat, but the effect of the composition of the diet on this characteristic was less conclusive. The molecular weight of carcass fat from rats which had subsisted on the three test diets was usually found to be greater than the calculated molecular weight of tristearin. The results of molecular weight determinations suggest that these fats contain significant amounts of fatty acids whose carbon chains are more than 18 carbons in length. These findings support the conclusions of Channon, Jenkins and Smith ('37).

SUMMARY

The effects of maintaining groups of young rats for 28 days on isocaloric food intakes (restricted) consisting of low-fat, 10% fat and 20% fat diets, supplemented with levels of thiamine ranging from 0 to 1,000 μg per day, were studied from the viewpoint of growth, cardiac thiamine, and amount and nature of carcass fat. The resultant data permitted the following conclusions:

1. The increase in body weight varied directly with the fat content of the diet and was not significantly affected by thiamine intake where the intake was 2 μg or more per day.

2. The weights of the fat-free carcasses were remarkably uniform for the rats from all experimental groups, indicating that the increments in weight were due primarily to deposited fat.

3. The cardiac thiamine varied directly with thiamine intake over a range of levels of from 0 to 1,000 μg per day and was not markedly affected by the fat content of the diet. Under the conditions of these studies maximum storage of thiamine in the heart did not appear to have been reached by those rats which received 1,000 μg of thiamine per day.

4. The deposition or retention of carcass fat, while dependent primarily on the fat content of the diet, was suppressed by extreme combinations of low-fat and low-thiamine or high-fat and high-thiamine dietaries. Rats receiving fat-containing thiamine-free diets require supplementary thiamine.

5. The index of refraction and the iodine number of the carcass fats decreased with an increase in the fat content of the diet and also with the amount of fat deposited by the rats constituting the respective dietary groups.

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THE THIAMINE REQUIREMENT OF THE ADULT RAT AND THE INFLUENCE ON IT OF A LOW ENVIRONMENTAL TEMPERATURE[1]

D. MARK HEGSTED AND GWELDA S. McPHEE

Department of Nutrition, Harvard School of Public Health, and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

TWO FIGURES

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Previous studies of the relation of thiamine to environmental temperature have been concerned with the effects of tropical temperatures. Mills and his co-workers (Mills, '41, '43; Mills, Cottingham and Taylor, '47) have presented consistent evidence that the thiamine need per gram of ration is elevated at high temperatures. Edison et al. ('45) and Kline et al. ('45), however, concluded that the requirement per gram of food was essentially the same at the two temperatures they studied. Various explanations have been advanced to account for the variation in results and to support the various experimental technics used. These need not be reviewed again. The problem remains of considerable practical and theoretical importance, since it appears to have been rather generally accepted that the thiamine requirement is proportional to the non-fat calories metabolized. If the con-

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clusion of Mills et al. is confirmed, this will not be applicable as a general proposition.

In the studies to be reported in this paper, concerned with thiamine requirements at low temperatures, several variables were encountered in the early work which required consideration. Young animals at 55°F. did not grow as well as at higher temperatures, their food intake was greater, and the incidence of respiratory infections was increased. It is known (Swift and Forbes, '39) that the metabolic rate is elevated at low temperatures. Therefore any of the above effects induced by a change in environmental temperature must be considered, until proven otherwise, as a possible factor influencing thiamine requirements in addition to the environmental temperature itself, although they may not be separable experimentally from the change in environmental temperature. It was felt that even though the requirement for maximum growth in the present studies was determined at two temperatures, the results could not be interpreted on any logical basis if this maximum rate was different at the two temperatures. All studies have, therefore, been done with adult animals. The advantages in the use of adult animals appeared to be that the rate of growth in response to thiamine was not different at the temperatures used, that the gain in weight was small in comparison to the total body weight and therefore the amount of thiamine required for the formation of new tissue was a minor variable, and that the adult animals were in general more resistant to the direct effects of low temperatures.

EXPERIMENTAL

Two separate studies are reported. In experiment I, adult male rats weighing approximately 200 gm were all placed in the cold room (55°F.) and fed a commercial dog food [2] for one week. It appeared wise partially to acclimatize all animals to the lower temperature, since some animals lose weight

[2] Purina Dog Chow.

during the first few days in the cold room. The opposite shift in environmental temperature is apparently made with ease.

After the preliminary adjustment period the animals were placed in individual cages at 55 or 78°F. and fed a purified thiamine-free diet of the usual type. It contained vitamin-free casein 18%, corn oil 5%, glucose 72%, cod liver oil 1%, and salt mixture 4 (Hegsted et al., '41) 4%. Crystalline vitamins were added to supply the following amounts per kilogram of diet: riboflavin 8 mg, nicotinic acid 40 mg, pyridoxine hydrochloride 4 mg, calcium pantothenate 25 mg, and choline chloride 3 gm. After one week of depletion on this diet, daily subcutaneous injections of thiamine were started. Twenty animals, 10 at each temperature, received 5 μg of thiamine chloride daily. Other groups of the same size at each temperature received 7 μg and 9 μg daily. Four representative animals in each group at each temperature were selected and their food consumption measured. Animals were weighed twice weekly. The study was continued for 7 weeks.

Experiment II was similar to experiment I, with the following exceptions: after the animals were placed in individual cages, they were given the purified diet containing excess thiamine (4 mg per kilogram of diet) for two weeks. All then received the thiamine-free diet until the average growth rate began to show a plateau. This required 9 days at 55°F. and 16 days at 78°F. It was hoped that this would represent equal depletion at the two temperatures. Eight groups of 8 animals each were used. Those at 78° received 5, 7, 9 and 50 μg of thiamine subcutaneously daily. Those at 55° received larger amounts — 8, 11.2, 14.5, or 50 μg per day — since it was apparent from experiment I that the requirement was somewhat elevated at the low temperature. This experiment was also continued for 7 weeks.

The temperature in the animal rooms was constant within 2°. The room air was exchanged rapidly and there was no control of humidity other than that provided by the precooling of the air as it entered the air conditioning unit.

RESULTS

It is convenient to discuss the results of the second experiment first, since in this study there were groups of animals which received excess thiamine and, therefore, should have shown maximum gain. In figure 1 the total gain in 7 weeks is plotted against the logarithm of the dose of thiamine per kilogram of body weight for each rat, using the weight of the animals at the time thiamine supplements were started.[3]

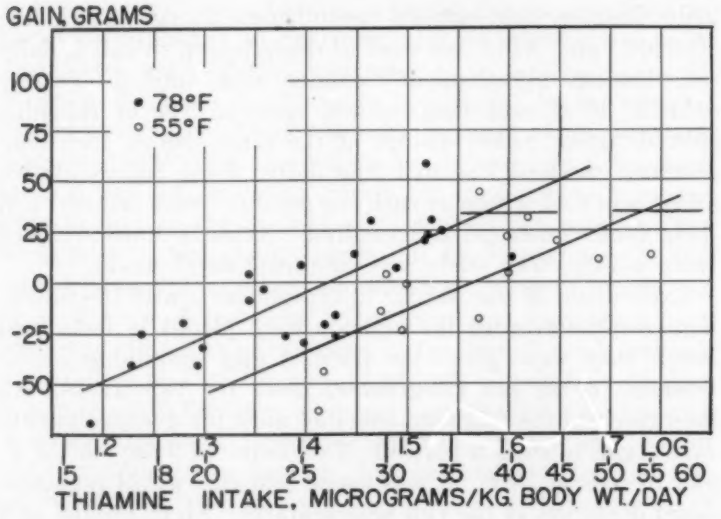

Fig. 1 The relation between the dose of thiamine and gain in experiment II.

As in previous studies (Hegsted, '48), the requirement is taken as the intersection of the regression line with a horizontal line representing maximum gain, in this instance the gain of the groups which received 50 μg of thiamine per day. It will be observed that the regression lines obtained at the two temperatures are essentially parallel, indicating that the thiamine requirement at the low temperature was a constant

[3] The animals in the group at 55°F. which received 14.5 μg of thiamine daily are not included since they showed maximum growth.

percentage above that at 78°. Maximum gain was essentially the same at both temperatures. The requirements read from the graph were 36.5 μg per kilogram of body weight per day at 78°F. and 53.5 μg at 55°F.

A similar plot of the data from experiment I is shown in figure 2. In order to calculate requirements on a comparable basis, it was necessary to assume that the animals in the low temperature room would have gained the same as the highest

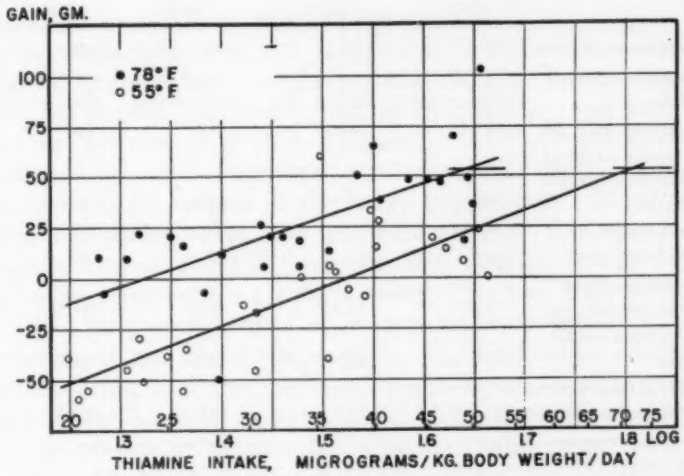

Fig. 2 The relation between the dose of thiamine and gain in experiment I.

level reached at 78°F. if sufficient thiamine had been provided. Since this was true in the second experiment, it does not appear to be a dangerous assumption. Maximum gain was therefore taken as the average of the group which received 9 μg at 78°F. This was 53 gm. Estimated requirements to give this gain were 49.3 μg per kilogram of body weight per day at 78°F. and 65 μg at 55°F.

Utilizing the data on food intake which were obtained prior to the development of deficiency with 24 animals in experiment I and 32 animals in experiment II, the calculations

shown in table 1 were made. The mean food intake was essentially the same per rat in the two studies, although the rats were somewhat larger in the second experiment. From the mean weight of the rats used, the non-fat calorie intake

TABLE 1

The effect of environmental temperature upon thiamine requirements

	EXPERIMENT I		EXPERIMENT II	
	78°F.	55°F.	78°F.	55°F.
Number of animals	29	28	24	14
Slope of regression line	166.0 ± 34.8 [1]	192.4 ± 32.1 [1]	218.5 ± 18.1 [2]	204.5 ± 60.8 [2]
Maximum gain, gm	53	(53) [3]	33.0	34.7
Thiamine intake at maximum growth, μg/kg	49.3	71.2	36.5	53.5
Mean weight of animals, gm	200	210	272	266
Requirement per rat per day, μg	9.87	14.95	9.93	14.22
Mean food intake, gm	16.5	20.2	16.2	20.5
Mean intake, non-fat calories per day	60.0	73.6	59.0	74.6
Thiamine requirement per 1,000 C., mg	0.164	0.203	0.168	0.191
Thiamine requirement per 100 gm non-fat food, μg	65.5	81.2	67.2	76.4

[1] Regression line of figure 2.
[2] Regression line of figure 1.
[3] Estimated.

and the estimated thiamine requirement per kilogram of body weight, the requirement on a calorie basis may be calculated. One obtains 0.164 and 0.168 mg of thiamine per 1,000 non-fat calories as the requirements at 78°F. and 0.203 and 0.191 mg per 1,000 as the requirements at 55°F.

DISCUSSION

It appears fairly clear from the data presented that thiamine requirements are raised by a decrease in environmental temperature. In the two studies, a drop in temperature of 23°F. resulted in an increase in thiamine requirement of 5.08 μg and 4.29 μg per rat per day. This represents an increase of approximately 50% (43 to 58%). Since the regression lines obtained in relating the logarithm of thiamine intake to gain were parallel, it would appear that this relation obtained at all doses of thiamine studied.

In comparison to a 50% increase in thiamine requirement per kilogram of body weight per day, the food intake was increased approximately 25% (22 to 26.5%),[4] or from 16.3 gm per rat per day at 78°F. to 20.3 gm at 55° F. Thus the data indicate a higher thiamine requirement per gram of food or per non-fat calorie at the lower temperature. Since this is a calculated value, one cannot determine the statistical significance of these differences with respect to the thiamine requirement on a calorie basis. The fact that the two studies were consistent in showing a higher requirement per calories at the lower temperature may lend some credence to the belief that the increased requirement is not entirely due to the increase in food intake. The possibility may be worth consideration that the thiamine requirement is proportional to the metabolic rate. We did not determine metabolic rates, but the data of Swift and Forbes ('39) indicate an increase of approximately 37% with this fall in temperature. This more nearly approaches the difference in thiamine requirements.

If thiamine is required in proportion to the non-fat calories metabolized, it is not clear why this should be true. One does not ordinarily suppose that catalysts are "worn out" as they function. Rather one would be led to suggest that thiamine or cocarboxylase is more susceptible to destruction in one form or combination than another and that an increase in

[4] Studies done in other experiments have confirmed this difference in food intake at the two temperatures.

its use as a coenzyme presents it in labile form or under circumstances in which it can be destroyed a greater percentage of the time. Thus one would have to consider not only the turn-over rate of the coenzyme but also the possibility of an increase in those factors causing its destruction. These might be independent variables.

It is of some interest to compare the values obtained in this study with those reported in the literature, since most previous work has been done with young animals. The summary presented by Brody ('45) for various species gives values ranging from 50 to 150 μg per 100 gm of diet. Our data yield approximate values of 65 μg per 100 gm of diet at 78°F. and 80 μg at 55°F. The data presented by Brown and Sturtevant ('49) indicate that the utilization of thiamine is about 15% better in the growing rat when it is administered parenterally rather than orally. Our figures might be expected, on this basis, to be somewhat below most of those previously reported. However, the effect of the route of administration requires additional study, since in the work reported by Brown and Sturtevant the animals fed orally did not gain as much as those fed parenterally even at very high doses of thiamine.

The available data for man have been critically reviewed by Holt ('44) and the conclusion is reached that "the minimum requirement for adult man lies at a point somewhere between 0.13 and 0.17 mg per 1,000 calories, whereas with a diet constructed of natural foods in which there is some variation both in the food and the thiamine intake, the minimum lies between 0.17 and 0.23 mg per 1,000 calories." Our values of 0.16 to 0.20 mg per 1,000 calories fall within this range. The thiamine requirements of the adult rat and adult man thus appear to be essentially the same when expressed on a calorie basis.

SUMMARY

The thiamine requirement of adult rats kept at 78°F. and at 55°F. was determined, using growth after partial depletion as the criterion of adequacy.

At the lower temperature the thiamine requirement per rat per day was 50% greater than at 78°F. Food intake data obtained prior to depletion showed that the animals at 55°F. were consuming approximately 25% more calories than those at 78°F. Calculation of the thiamine requirement on the basis of the non-fat calories consumed gave values of 0.164 to 0.168 mg per 1,000 non-fat calories at 78°F. compared to 0.191 to 0.203 mg at 55°F.

The increased food intake at low temperatures thus accounts for much, but may not account for all, of the increased thiamine requirement.

The thiamine requirement of the adult rat per 1,000 non-fat calories consumed appears to be essentially the same as the requirement of adult human beings on the same basis.

ACKNOWLEDGMENTS

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CALCIUM RETAINED FROM ONE LEVEL OF INTAKE BY SIX ADOLESCENT GIRLS

FRANCES A. JOHNSTON, DORETTA SCHLAPHOFF[1] AND THELMA McMILLAN

New York State College of Home Economics and the School of Nutrition, Cornell University, Ithaca

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In a survey of the literature, calcium balance studies on 42 girls in the age range of 12 to 15 years, or who were younger but had passed the menarche, were found. These studies were made by Sherman and Hawley ('22), Willard and Blunt ('27), Wang, Kaucher and Frank ('28), Wang, Kaucher and Wing ('36), Johnston ('40, '41) and McKey, Smith and Smith ('48). These investigators have made important contributions in the development of the present concept of calcium needs. The daily dietary allowance recommended by the Food and Nutrition Board of the National Research Council ('48) for girls 13 to 15 years of age is 1.3 gm. The recommendation is accompanied by a statement that "data on which to base requirements were meager." Because of the need for more data, an iron balance study in our laboratory on 6 adolescent girls (Schlaphoff and Johnston, '49) was extended to include calcium as well.

PROCEDURE

The subjects were 6 girls 13 and 14 years of age whose heights ranged from 60⅝ in. to 67⅝ in. (table 1). Because of the probable effect of stores on calcium retention, only girls whose dietary history showed they had been drinking

[1] Present address: Department of Home Economics, College of Agriculture, University of Nebraska, Lincoln.

at least three glasses of milk a day were accepted as subjects. Because the experiment was carried on during July and August when the subjects were out of doors in the sunshine much of the time, a concentrated source of vitamin D was not given.

The subjects were fed for 9 weeks on a carefully controlled diet made up of a wide variety of common foods. During the entire time the calcium intake for each girl was approximately 1.0 gm per day. Each meal included 182 gm of milk, making a total of 546 gm or two and one-quarter cups for

TABLE 1

Growth and menarcheal data for the 6 subjects

SUBJECT	AGE [1]	AGE AT MENARCHE		LAPSE OF TIME SINCE MENARCHE [2]	HEIGHT [3]	GROWTH IN 9 MO. [4]
	yr.	*yr.*	*mo.*	*months*	*in.*	*in.*
A	14	13	8	6	64½	½
B	14	12	11	8	62	⅜
C	13	10	? [5]		64¼	¼
D	14	13	6	2	61¾	1½
E	13	12	11	6	60⅜	⅜
F	13	12	8	1	67⅛	1½

[1] Age to nearest birthday.
[2] Until the beginning of the experiment.
[3] At the beginning of the experiment.
[4] From the beginning of the experiment.
[5] The exact month at which the menarche occurred is not known.

the day. In addition, the diet contained milk used in cooking, whipping cream, cheese and ice cream. About nine-tenths of the calcium of the diet came from milk and milk products. Other details of the diet are to be found in the previous paper (Schlaphoff and Johnston, '49). Changes made in the foods in the diet at the end of 5 weeks in order to increase the iron content increased the calcium content by 65 mg. Bread, cookies, jelly, butter, sugar, and candy made with water were allowed ad libitum and a limited amount of a cola drink was permitted. Distilled water was used for cooking and drinking.

Stools and urine were collected for 8 weeks following a one-week adjustment period. The preparation for analysis of the foods, stools and urine, as well as the ashing of the foods and stools, are described in the previous paper. The urine was analyzed without preliminary ashing. Analyses for calcium were made by the McCrudden method ('11).

RESULTS AND DISCUSSION

The mean calcium intake for the 6 subjects for 8 weeks was 1,079 mg per day and ranged from 1,050 to 1,131 mg (table 2). The difference of 65 mg between the intake of the first and second 4-week periods was found by the method of covariance to have made no significant difference in the amount retained. The minor variations in intake from week to week and from subject to subject within each 4-week period were due mainly to the calcium contained in the foods allowed ad libitum. The mean amount retained for 8 weeks was 281 mg per day and the range was from 174 to 444 mg (table 2). In terms of percentage, the mean amount of calcium retained from the intake was 26% and the range was from 16 to 41%. This mean is similar to the mean of 29% found by Johnston ('40, '41) for 7 girls 9 to 15 years of age who had passed the menarche and whose calcium intake ranged from 1,104 to 1,488 mg per day. It is similar, also, to the mean of 24% found by McKey, Smith and Smith ('48) for 7 girls 12 to 13 years of age whose mean intake of calcium was 935 mg per day. The results of this study extending over 8 weeks show that the shorter period of 18 or 20 days used in the other two studies was sufficient.

The reasons for variation in the amount of calcium retained by different subjects on the same intake are little understood. Among the possible causes are: the need, which may be affected by the stores and the rate of calcification; the supply of vitamin D; and the amount of glandular secretions. The effect of calcium stores on retention was demonstrated by Fairbanks and Mitchell in 1936 and by Rottensten in 1938, who showed that, on a diet of a given calcium content, rats

TABLE 2

The amount of calcium ingested, excreted and retained by 6 girls 13 and 14 years of age

SUBJECT		WEEK I	WEEK II	WEEK III	WEEK IV	WEEK V	WEEK VI	WEEK VII	WEEK VIII	WEEK IX	MEAN	
		mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	%
A	Ca in food	1052 [1]	1040	1036	1053	1039	1105	1086	1106	1106	1071	
	Ca in feces		675	651	643	676	680	758	738	764	698	
	Ca in urine		60	69	77	80	85	87	87	70	77	
	Ca retained		305	316	333	283	340	241	281	272	296	28
B	Ca in food	1043	1040	1020	1028	1013	1083	1051	1078	1084	1050	
	Ca in feces		792	505	540	740	750	869	674	871	718	
	Ca in urine		86	87	90	91	102	104	85	101	93	
	Ca retained		162	428	398	182	231	78	319	112	239	23
C	Ca in food	1051	1048	1036	1056	1034	1117	1087	1133	1120	1079	
	Ca in feces		701	704	666	763	778	738	772	735	733	
	Ca in urine		145	165	170	189	208	194	176	128	172	
	Ca retained		202	167	220	82	131	155	185	257	174	16
D	Ca in food	1052	1049	1041	1082	1077	1131	1091	1127	1132	1091	
	Ca in feces		460	418	476	699	603	533	556	524	534	
	Ca in urine		76	97	123	113	122	119	144	113	113	
	Ca retained		513	526	483	265	406	439	427	495	444	41
E	Ca in food	1039	1036	1020	1025	1009	1085	1059	1078	1084	1050	
	Ca in feces		608	629	654	578	656	611	650	636	628	
	Ca in urine		112	153	143	129	158	152	150	150	143	
	Ca retained		316	238	228	302	271	296	278	298	279	27
F	Ca in food	1057	1062	1066	1115	1111	1201	1158	1183	1156	1131	
	Ca in feces		638	649	704	672	724	724	717	668	687	
	Ca in urine		147	244	234	184	228	178	188	165	196	
	Ca retained		277	173	177	255	249	256	278	323	248	22
Mean	Ca in food										1079	
	Ca in feces										666	
	Ca in urine										132	
	Ca retained										281	26

[1] Values for week I are not included in the average.

with poor stores retained more than rats with good stores. Subsequently, Macy ('42) found this to be true for boys 4 to 6 years of age, but Breiter et al. ('41) failed to find a similar relationship with adult subjects. The effect of vitamin D on the retention of calcium is well established. Amounts of estrogen above normal decrease the amount of calcium retained by adolescent girls (Johnston, '41) and either subnormal or excessive amounts of thyroid reduce the amount of calcium retained (Johnston and Maroney, '39).

In the present study subjects A, B, E and F retained amounts near the mean; subject C retained much less and subject D much more than the mean. The reason for the variation among these subjects is not known. A difference in stores was probably not the reason, as only girls who had been drinking three glasses of milk a day were accepted as subjects. Possibly the rate of calcification was the most important factor. The low retention of calcium by subject C may have been due to a low rate of calcification, as she had passed the prepubertal growth period two years (table 1) before the beginning of the experiment. For subject D the high retention may have been due to a rapid rate of calcification; she was growing rapidly (table 1). With human subjects an interplay exists among so many factors that it is probably over-simplification to say that any one cause is solely accountable for the differences in the amounts retained.

Little variation in the fecal excretion of calcium occurred from week to week except in the case of subject B. The irregularity in excretion for that subject may have been apparent and not real; it may have been due to errors in stool separation. Carmine given this subject early Monday morning usually did not appear until Thursday or Friday; during this wide lapse of time the carmine may have, in some weeks, spread forward as well as backward. Sometimes emotional disturbances are thought to be the cause of a temporary decrease in the amount of calcium absorbed. For this subject no correlation was noted between emotional stress and short periods of poor absorption.

Mean values for each of the 6 subjects for the urinary excretion of calcium for 8 weeks ranged from 77 to 196 mg per day (table 2). Although this is a wide range, all values fell within the range considered normal by Knapp ('47). The wide range for normal excretion is due, in the opinion of Knapp, to "an endogenous factor or factors, presumably endocrine."

TABLE 3

An estimation of the amount of calcium which needs to be retained by adolescent girls

AGE	WEIGHT GAIN			ESTIMATED DAILY CALCIUM ACCRETION
	Yearly	Daily	Daily % of total accretion	
yr.	*kg*	*gm*	%	*mg*
	Early menarche (10 yr. to 11 yr. 11 mo.)			
11	7.04	19.29	0.0367	319
12	3.73	10.22	0.0194	168
13	2.00	5.48	0.0104	90
14	1.50	4.11	0.0078	68
	Medium menarche (12 yr. to 12 yr. 11 mo.)			
11	6.14	16.82	0.0320	278
12	5.50	15.07	0.0286	248
13	4.18	11.45	0.0218	189
14	2.00	5.48	0.0104	90
	Late menarche (13 yr. to 15 yr. 5 mo.)			
11	3.86	10.57	0.0201	175
12	5.50	15.07	0.0286	248
13	5.09	13.94	0.0265	230
14	3.41	9.34	0.0178	155

The question may be raised in regard to all calcium balance experiments of whether calculation of the amount retained is fictitiously high because of failure to account for losses of calcium in hair, skin cells, perspiration and menses, and to collect all the fecal excretion. In the present study these losses were not measured, but the entire fecal excretion was collected. High-grade paper handkerchiefs were used in

place of toilet paper and were added to the composite. They were analyzed for calcium and found to contain so little that it fell within the experimental error.

The adequacy of the daily intake of 1 gm of calcium for the 6 subjects of this study may be judged by comparing the amount retained with estimates of the amount of calcium which needs to be retained for the building of new bone and tissue at this age. In 1945 Holmes reviewed estimates for children made by several other workers and set up one of her own for boys. We have applied her experimental plan to adolescent girls. In general outline the plan is to find the total increment of calcium from birth to adulthood and to apportion the increments during growth to correlate with weight increments. Holmes states that "substantial evidence exists that the calcium content of the newborn averages 0.8%." She states that few data can be found upon which to base a value for the calcium content of the properly calcified adult. After a discussion of the available data she concludes that the best estimate which can be made at present is 1.60%. Holmes also presents evidence to show that increments of calcium from year to year during growth are almost identical with those in body weight.

In making an estimate for girls, the birth weight of the average healthy baby girl was taken as 3.4 kg and the weight of the adult woman as 56 kg. The calcium content at birth was taken as 0.8% and in adulthood as 1.60%. Because the age at which accelerated growth occurs is related to the age at menarche, estimates for girls with early, medium and late menarches were made. The yearly weight gains found by Simmons and Greulich ('43), who studied the growth of a group of 200 adolescent girls, were used. The resulting values are given in table 3.

Of the subjects of this study, one fell in the early, three in the medium and two in the late menarcheal group. All retained much more calcium on an intake of approximately 1 gm than the estimated amount required (table 3). It is interesting to note that, according to this estimate of the amount which

needs to be retained, subject C, who retained the least, needed the least and that subject D, who retained the most, needed the most. All subjects of this study had passed the age of the greatest estimated required retention. To find the maximum requirement during adolescence, possibly girls of ages 11 and 12 years should be studied.

All the subjects of this study were eating a diet carefully planned to be adequate in all known nutrients. McKay et al. ('42) found that more calcium was retained from such a diet than from a poor one. Thus, these subjects might have retained less on a diet less adequate with respect to other nutrients.

An estimate of the amount of calcium absorbed can be derived from the data collected in this experiment. Whether or not calcium passes from the blood plasma into the intestinal lumen through the intestinal wall is a question. Evidence that this does not occur has been found by McCance and Widdowson ('39), who discovered no increase in calcium in the gastrointestinal tract after injecting calcium into the blood stream. The opposite view is held by Steggarda and Mitchell ('46), who have reviewed the evidence supporting the theory that calcium does pass through the intestinal wall into the tract. If the theory that calcium does not pass through the wall is the correct one, then the amount absorbed can be found by subtracting fecal calcium from food calcium and correcting for the calcium in the digestive secretions. The mean value found by subtracting fecal calcium from food calcium for the present 6 subjects for each 4-week period was 38.2%; the range was from 26.3 to 51.1% (table 4). To arrive at a true value for absorption, these figures would have to be raised somewhat to account for calcium from the digestive secretions.

This experiment afforded an opportunity for comparing the percentage absorption of calcium with that of iron, because the same foods and stools were analyzed for both elements. A comparison of the two is of interest because both form many insoluble compounds which, if present as such in

the upper part of the gastrointestinal tract, cannot be absorbed. The mean absorption of iron was 14.0%; the range was 3.4 to 19.6% (table 4). Thus, calcium absorption was about three times greater than iron absorption. The percentage absorption of calcium was quite even for all the subjects except subject B, while the absorption of iron (table 4) varied widely.

TABLE 4

The percentage of calcium and iron absorbed from the diet[1]
(Food content minus fecal content)

WEEK	Fe	Ca	Fe	Ca	Fe	Ca
	Subject A		Subject B		Subject C	
II	8.7	35.1	8.3	23.8	17.2	32.9
III	15.4	37.2	35.1	50.4	15.7	32.1
IV	11.2	38.9	31.7	47.4	19.7	36.9
V	4.5	34.9	1.8	27.0	7.7	26.3
Mean	10.0	36.5	19.6	37.1	15.2	32.1
VI	16.3	38.5	6.5	30.7	12.2	30.4
VII	10.0	30.2	1.9	17.3	19.1	32.1
VIII	9.6	33.3	6.6	37.4	13.7	31.9
IX	6.3	31.0	— 1.5	19.6	10.4	34.4
Mean	10.5	33.2	3.4	26.3	13.9	32.2
	Subject D		Subject E		Subject F	
II	18.0	56.2	23.3	41.3	— 1.5	39.9
III	26.2	59.9	16.7	38.4	14.0	39.2
IV	21.3	56.0	12.6	36.2	13.7	36.9
V	— 8.4	35.1	21.1	42.8	11.1	39.5
Mean	14.1	51.7	18.4	39.7	9.7	38.9
VI	16.5	46.7	17.5	39.6	18.5	39.7
VII	25.0	51.1	21.2	42.3	20.3	37.5
VIII	19.3	50.7	13.5	39.7	7.7	39.4
IX	15.4	53.7	15.3	41.3	19.8	42.3
Mean	19.1	50.5	16.8	40.7	16.5	39.7

[1] No correction has been made for calcium or iron in the digestive fluids.

SUMMARY

Six girls 13 and 14 years of age were maintained for 9 weeks on a controlled diet. The total period was divided into a one-week adjustment period and an 8-week experimental period. During the entire time the diet contained ap-

proximately 1 gm of calcium per day. The mean amount of calcium retained by each of the 6 subjects was 174, 239, 248, 279, 296 and 444 mg per day. The mean retention was 281 mg, or 26% of the intake.

On the basis of the limited data available an estimate of the amount of calcium which needs to be retained by girls of this age for the building of bone and other tissue was made. As judged by this estimate, each of the 6 subjects was retaining an adequate amount.

ACKNOWLEDGMENTS

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EFFECT OF A HIGH LEVEL OF METHIONINE ON THE DIETARY CHOLINE REQUIREMENT OF THE BABY PIG

R. O. NESHEIM AND B. CONNOR JOHNSON
Division of Animal Nutrition, University of Illinois, Urbana

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It has been established that the baby pig requires choline (Johnson and James, '48a) and that the amount required is approximately 0.1% of the dry matter of the diet when the diet contains 0.8% to 1.0% methionine (Neumann et al., '49). The present experiment was designed to study the effect of a high level (1.6%) of methionine in the diet on the dietary choline requirement of the baby pig.

EXPERIMENTAL

Twelve three-day-old Duroc pigs from the University farm [1] were used in this study. The care and feeding of the animals have been described elsewhere (Johnson, James and Krider, '48b). The composition of the basal diet is given in table 1. Liver extract [2] was injected at the rate of 0.1 ml per pig daily to supply vitamin B_{12} and possibly unidentified factors. Two grams of sulfathalidine [3] were fed per liter of diet to inhibit intestinal synthesis.

The pigs were divided into 6 pairs and pair-fed equal feed intakes for 56 days. One pig of each pair received the basal

[1] We wish to acknowledge the generosity of Dr. J. L. Krider of the Swine Division in making available the newborn pigs used in this study.

[2] The liver extract, Lederle 15-unit injectible, was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

[3] Sulfathalidine was supplied by Sharp and Dohme, Inc., Glenolden, Pennsylvania, through the courtesy of Dr. S. F. Scheidy.

TABLE 1

Composition of the basal diet

CONSTITUENTS [1]	AMOUNT	VITAMINS [2] ADDED PER LITER OF "MILK"	AMOUNT
	%		
Casein (Labco,		Vitamin A	2,000 I.U.
vitamin-free)	28.2	Vitamin D	200 I.U.
DL-methionine	0.8	α-Tocopherol acetate	1.0 mg
Lard	29.0	2-Methyl-1,4-naphthoquinone	0.26 mg
Glucose (Cerelose)	36.0	Thiamine	0.65 mg
Mineral salts [3]	6.0	Riboflavin	1.33 mg
		Nicotinic acid	2.67 mg
		Pyridoxine	2.67 mg
		Ca-pantothenate	8.00 mg
		Inositol	26.00 mg
		p-Aminobenzoic acid	2.67 mg
		Folic acid	0.052 mg
		Biotin	0.01 mg
		Ascorbic acid	10.00 mg

[1] These ingredients were made into a "milk" (13% solids) containing 4% lard (liquid basis).

[2] Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin, nicotinic acid, ascorbic acid and alpha-tocopherol acetate were supplied by Hoffmann-LaRoche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind. Folic acid was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes. DL-methionine was supplied by the Dow Chemical Co., Midland, Michigan, through the courtesy of Dr. J. E. Johnson. Water-soluble emulsions of vitamins A and D (A-CON and D-CON) were supplied by Endo Products, Inc., Richmond Hill, New York, through the courtesy of Dr. S. M. Gordon.

[3] See Johnson et al. ('48b).

diet; the other received the basal diet plus 0.1% (dry basis) choline chloride. By analysis the basal diet contained 1.6% methionine, with 0.8% supplied by the casein and 0.8% added as DL-methionine. At the close of the feeding trial 5 of the pairs were slaughtered, and histological examinations [4] were made of sections of the livers and kidneys.

[4] We wish to acknowledge the help of Dr. Marian F. James in carrying out these examinations.

RESULTS AND DISCUSSION

The average performance records for the 6 pairs of pigs are given in table 2. There were no statistically significant differences in rate of gain or efficiency of feed utilization between the members of the pairs. Histological examination of the livers and kidneys showed all to be normal, with no differences between the members of the pairs. Thus it appears that the pig even at this early age is able to use methionine as a methyl donor in the synthesis of choline. In this respect the pig resembles the rat (Treadwell, '48) and man (Simmonds and du Vigneaud, '42), but may differ from the chick (Jukes, '40; McKittrick, '47). The baby pig does not require dietary choline when the diet contains 1.6% methionine.

TABLE 2

Effect of choline on growth and feed efficiency when the diet contained 1.6% methionine (12 pigs pair-fed for 56 days)

	BASAL DIET (1.6% methionine)	BASAL DIET + 0.1% CHOLINE CHLORIDE
Number of pigs	6	6
Average initial weight, kg	1.9	1.9
Average final weight, kg	21.2 (46.7 lb.)	21.2 (46.5 lb.)
Average: $\frac{\text{final weight}}{\text{initial weight}}$	10.9	11.0
Kilogram dry matter consumed/kg gain	1.17	1.15

CONCLUSION

On a synthetic milk diet containing 1.6% methionine the baby pig exhibited no demonstrable dietary choline requirement.

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THE ACTIVITIES OF D AND L LYSINE FOR TURKEY POULTS

F. H. KRATZER
Division of Poultry Husbandry, University of California, Davis

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It has been demonstrated that growing turkeys require lysine in their ration for optimum growth and for normal feather pigment formation (Fritz et al., '46; German et al., '49; Kratzer et al., '50). Since the unnatural isomers of some amino acids are not active and others have limited activity when fed to avian species (Almquist, '48), it was of interest to determine the value of D-lysine for the turkey poult.

EXPERIMENTAL

The lysine-deficient ration which was used in the present experiment contained the following in grams per 100 gm: sesame seed oil meal, 57.0; cornstarch, 24.1; calcium gluconate, 5.0; oil mixture, 3.5; salt mixture, 2.5; vitamin mixture,[1] 2.0; condensed fish solubles,[2] 2.0; calcium phosphate (tribasic), 2.0; calcium phosphate (dibasic), 1.5; choline chloride, 0.3; inositol, 0.1; and biotin, 0.00002. The oil mixture supplied soybean oil, 2.5 gm; fish oil (2,000 A, 400 D), 1.0 gm; and alpha tocopherol acetate, 0.002 gm. The salt mixture and vitamin mixture were essentially those described previously (Kratzer et al., '49). Additions of lysine were made at the expense of cornstarch. The basal ration contained approximately 25%

[1] Pteroylglutamic acid was kindly donated by the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

[2] Kindly donated by the Valley Dehydrating Company, Kingsburg, California, through the courtesy of Mr. E. B. Fishel.

TABLE 1

The effect of various forms of lysine on the growth and feather pigmentation of poults

SUPPLEMENT	LEVEL	AVERAGE DAILY GAIN			AVE. OF ALL EXPTS.	INCIDENCE OF WHITE BAR	
		Expt. 1	Expt. 2	Expt. 3		Expt. 1	Expt. 2
	%	%	%	%	%	%	%
None		3.3	3.2	2.9	3.1	100	86
L-lysine monohydrochloride	0.25	5.1	5.1	4.5	4.9	83	71
L-lysine monohydrochloride	0.5	6.6	6.4	6.1	6.4	0	0
L-lysine monohydrochloride	0.75	7.8	7.0		7.4	0	0
DL-lysine monohydrochloride	0.25	4.0	3.2		3.6	71	86
DL-lysine monohydrochloride	0.5	5.7	4.1	4.1	4.6	100	57
DL-lysine monohydrochloride	0.75	5.4	5.2		5.3	17	25
DL-lysine monohydrochloride	1.00			6.3	6.3		
D-lysine monohydrochloride	0.25		2.8		2.8		86
D-lysine monohydrochloride	0.5		3.3		3.3		100
D-lysine monohydrochloride	0.75		2.9		2.9		83
L-lysine monohydrochloride	0.5			6.2	6.2		
D-lysine monohydrochloride	1.0						

protein. The lysine [3] samples used were commercial preparations.

Bronze turkey poults were fed a practical starting ration for from 8 to 16 days before they were divided into groups of

[3] Kindly donated by E. I. DuPont de Nemours and Company, Inc., New Brunswick, New Jersey, through the courtesy of Dr. J. Waddell.

7 each and were fed the experimental rations. The first three trials continued for 14 days, 16 days and 13 days successively. The poults were weighed and examined at frequent intervals. The results of the three trials are summarized in table 1. A high incidence of pigmentation failure was caused by feeding the basal ration, which was deficient in lysine. The incidence was reduced by 0.25% L-lysine monohydrochloride and the failure was entirely prevented by 0.5% L-lysine monohydrochloride. DL-lysine monohydrochloride was less effective and even 0.75% did not entirely prevent the pigmentation failure; D-lysine monohydrochloride at a level of 0.75% was ineffective in reducing the incidence of faulty pigmentation.

TABLE 2

The effect of D-lysine added to a practical poult starting ration upon the growth of poults

SUPPLEMENT	LEVEL	AVERAGE DAILY GAIN [1]
	%	%
None	..	5.65
D-lysine monohydrochloride	0.5	5.34
D-lysine monohydrochloride	1.0	5.22
D-lysine monohydrochloride	2.0	4.50

[1] Least difference for significance at 1% level = 0.61.

The growth rates of the poults increased directly as the L-lysine of the ration increased (table 1). Approximately twice as much DL-lysine was required to produce equivalent gains. D-lysine caused no increase in gain above that observed in poults fed the basal ration.

D-Lysine was tested in an adequate ration in experiment 4 to determine whether it would depress growth at relatively high levels. Various amounts of D-lysine were added to a practical poult starting ration, as is indicated in table 2. Eight poults per group were fed the rations for 18 days. There was no significant reduction in growth when the D-lysine was fed at 0.5% and 1.0%. At 2% of the ration growth was depressed significantly, as determined by the analysis of variance method (Snedecor, '46).

DISCUSSION

L-Lysine has been previously shown to be required by the turkey poult for normal growth and for feather pigment formation (Fritz et al., '46; German et al., '49; Kratzer et al., '50). The present work shows that the D-isomer of lysine cannot replace the natural form for these functions. The complete mechanisms by which lysine is used for growth or in the formation of melanin are not known. These two requirements have similar specificity for the isomers of lysine.

There is no indication in the results of these experiments that levels up to 1% of D-lysine are growth inhibiting; however, growth was depressed by 2% D-lysine. Prescott et al. ('49) have shown that D-tryptophan slightly inhibits growth in certain bacteria when it is supplied at relatively low levels.

The turkey poult, like the rat and mouse, is unable to use the D-isomer of lysine for growth (Berg, '36; Totter and Berg, '39).

SUMMARY

Turkey poults are unable to use D-lysine for growth or for the development of normal pigment in the feathers. Levels of D-lysine up to 1% did not depress growth, while 2% caused decreased growth when added to an adequate ration.

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UTILIZATION OF NIACIN PRECURSORS AND DERIVATIVES BY THE RAT AND NEUROSPORA [1]

W. A. KREHL, DAVID BONNER AND CHARLES YANOFSKY
Yale Nutrition Laboratory, Department of Physiological Chemistry, and Osborn Botanical Laboratory, Yale University, New Haven, Connecticut

ONE FIGURE

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INTRODUCTION

It has been well established that a deficiency of niacin in the diet can be met by an increased intake of tryptophan (Krehl et al., '46b) and that tryptophan promotes an increase in the urinary output of niacin derivatives (Rosen et al., '46; Singal et al., '46). Numerous studies with *Neurospora* mutants (Beadle et al., '47; Bonner, '48; Mitchell and Nyc, '48a) have established a metabolic pathway from tryptophan to niacin which has been shown to obtain in animal tissue as well (Heidelberger et al., '49). Of these intermediates (kynurenine, 3-hydroxy kynurenine, 3-hydroxyanthranilic acid), only 3-hydroxyanthranilic acid has shown distinct growth-promoting properties for the rat (Mitchell et al., '48b). Henderson ('49a) identified quinolinic acid as the acid labile compound in urine which Singal et al. ('46) first reported to have niacin activity for *L. arabinosis* after the urine was autoclaved in an acid medium. Quinolinic acid has been reported to lead to N^1-methylnicotinamide excretion (Ellinger et al., '47); to have slight effectiveness in relieving niacin

[1] Supported by grants from the National Vitamin Foundation, New York, New York and the Williams-Waterman Fund for the Combat of Dietary Diseases.

deficiency in the rat (Henderson, '49b); and to be formed from 3-hydroxyanthranilic acid by rat liver preparations (Henderson and Ramasarma, '49d; Schweigert and Marquette, '49). The administration of tryptophan has also been shown to lead to the urinary excretion of quinolinic acid by man and other mammals (Henderson et al., '49e).

The work reported here was concerned with further evaluation of the intermediates involved in the conversion of tryptophan to niacin and their role in overcoming niacin deficiency in the rat.

TABLE 1

Composition of niacin-deficient diets

COMPONENT[1]	DIETS			
	S	A	B	C
	%	%	%	%
Sucrose	81.8	75.8	...	...
Dextrin	...	...	81.8	75.8
Casein	9.0	9.0	9.0	9.0
Gelatin	..	6.0	..	6.0
Salts IV	4.0	4.0	4.0	4.0
Corn oil	5.0	5.0	5.0	5.0
Cystine	0.2	0.2	0.2	0.2

[1] Vitamins were added per kilogram of diet as follows: B_1, B_2, and B_6 10 mg each; calcium pantothenate 100 mg; biotin and folic acid 1.0 mg each; inositol and *p*-aminobenzoic acid 200 mg each; choline 1 gm; 2-methylnaphthoquinone 10 mg; vitamin D_3 100 μg; and vitamin A 30,000 I.U.

EXPERIMENTAL

Rat studies

Basal diet. The niacin-deficient diets used were similar in composition to those described by Krehl et al. ('46a) and their composition is given in table 1.

Procedure. Weanling male rats of the Sprague-Dawley strain were housed in dormitory style cages in an air-conditioned animal room which was maintained at a temperature of 26°C. with a relative humidity of 55%. The animals were fed and watered ad libitum and the standard procedure was to place the 21-day-old weanling animals on the niacin-defi-

cient diets containing 9% casein and 6% gelatin (diet A or C, table 1) for a period of three weeks, at which time their weight curves had reached a plateau.

Most of the rats at this stage showed other gross manifestations of niacin deficiency, i.e., porphyrin caked whiskers, some alopecia, unsteady gait and irritability. At this time the various test compounds were given in a treatment schedule which involved daily administration of the test material for a 5-day period (in certain noted exceptions a shorter or longer period was used). The test compounds were given either by intraperitoneal injection (I.P.) or as oral supplements in small supplement dishes. The animals were weighed every day from the start of the dosage period, and a time arbitrarily chosen as 10 days after initiating treatment was taken as a point for comparing weight responses obtained with the various compounds given.

Neurospora crassa studies

The details of these experiments are given below in the presentation of the results.

Enzyme studies

These involved experiments with liver tissue from adult rats. The details of the experiments are best presented along with the results (see below).

RESULTS

Rat growth studies

The weight gains during the three-week deficiency period, the number of rats used per test, the mode and kinds of treatment and the weight gain responses to the treatments are shown in table 2. In general, marked differences in the behavior of the rats on the sucrose diets S and A and the dextrin diets B and C are evident, as has been noted before (Krehl et al., '46a, b). The three-week weight gain on niacin-

deficient dextrin diet C was markedly better than on sucrose diet A (i.e., about 25 vs. about 5 gm). In addition, the response to added niacin of the animals fed the dextrin diets was always considerably greater than that of rats on the comparable sucrose diet (i.e., 50 gm in 10 days vs. 28 gm).

TABLE 2

Growth response to niacin precursors and derivatives

DIET USED	NO. OF RATS	AVE. WEIGHT GAIN IN 3 WEEKS	MODE OF TREATMENT	AVE. WEIGHT GAIN DURING 10 DAYS AFTER START OF TREATMENT
		gm		gm
S	12	27.0	Continue diet	8.6
A	6	6.1	1 ml phosphate buffer pH 7.3 I.P. for 5 days	2.0
B	9	77.0	Continue diet	29.3
C	6	25.0	Continue diet	7.6
A + 5 mg % niacin	6	44.7	Continue diet	15.2
A + 100 mg % anthranilic acid	6	3.2	Continue diet	1.2
A	5	5.8	1 mg niacinamide I.P. for 5 days	28.0
C	4	27.0	1 mg niacinamide I.P. for 5 days	50.0
A	5	6.2	10 mg L-tryptophan I.P. for 5 days	23.4
A	5	4.2	10 mg 3-hydroxyanthranilic acid I.P. for 5 days	20.4
A	5	8.7	10 mg L-kynurenine I.P. for 5 days	5.6
A	5	8.3	10 mg kynurenic acid I.P. for 5 days	−2.8
A	6	5.4	2 mg % quinolinic acid in diet — fed for 5 days	−8.7
A	5	7.2	2 mg % niacinamide in diet — fed for 5 days	21.0
A	5	6.4	5 mg % nicotinamide methylchloride in diet fed for 5 days	1.7
A	5	6.0	5 mg % 3-cyano pyridine in diet fed 5 days	−0.7
A	5	7.2	100 mg % acetyl kynurenine in diet fed 5 days	2.0
C	4	25.0	5 mg N-formyl kynurenine — orally for 5 days	4.0
C	3	21.0	5 mg N-formyl anthranilic acid — orally for 5 days	9.0
C	3	20.0	5 mg L-tryptophan orally for 5 days	42.0
C	3	21.0	5 mg DL-tryptophan orally for 5 days	32.0
C	4	22.0	1 mg quinolinic acid I.P. for 10 days	24.0
C	4	26.2	2 mg quinolinic acid orally for 10 days	20.0
C	3	23.1	0.2 mg niacinamide orally for 5 days	38.0

Because of these differences between sucrose and dextrin diets most of the compounds studied were tested with both types of diet (see table 2 for results).

Niacin-deficient rats (diet A) that were given, respectively, 1 mg of niacinamide, 10 mg of L-tryptophan and 10 mg of 3-hydroxyanthranilic acid intraperitoneally every day for 5 days showed growth responses of comparable magnitude, although the response obtained with niacinamide was better than that with L-tryptophan and 3-hydroxyanthranilic acid. A similar group of rats given 1-ml injections of phosphate buffer, pH 7.3, intraperitoneally for 5 days showed no significant growth response and no unfavorable effects as a result of the injections, and were used as controls for the groups injected with the various compounds.

It is quite interesting to note that the administration of kynurenine failed to elicit a significant weight increase in niacin-deficient rats, although this compound is thought to be one of the intermediates for *Neurospora* in the conversion of tryptophan to niacin (Beadle et al., '47; Yanofsky and Bonner, '50). This compound has been tested several times on different groups of rats, both by oral administration and by intraperitoneal injection, and in no case has a significant growth response been obtained.

Kynurenic acid and anthranilic acid were again, as previously reported (Krehl et al., '47), inactive in correcting niacin deficiency in the rat. Indeed it is worthy of note that animals which received anthranilic acid incorporated in the diet at a level of 100 mg% actually showed earlier and more advanced gross symptoms of niacin deficiency than any other group of animals.

As might be expected, both L- and DL-tryptophan induced rapid and large growth responses, although 5 mg of DL-tryptophan given daily for 5 days were not as effective in promoting growth as was L-tryptophan given in a similar dosage schedule. This dosage of tryptophan, in our experience, probably represents the minimum amount that will provide a maximum growth response, and is of course at least 25 to 50 times

larger than the amount of niacin needed to produce a comparable response.

Since Knox et al. ('49) reported that a system of liver enzymes converts tryptophan to a kynurenine-like compound, and because it was reported that the conversion of tryptophan to kynurenine required the removal of a carbon atom that was found as formic acid upon hydrolysis of the reaction product, N-formyl derivatives of kynurenine and anthranilic acid were prepared and tested for their growth activity. Neither of these two formyl derivatives proved active.

No activity in promoting growth could be found for N^1-methylnicotinamide or 3-cyanopyridine, as might be anticipated, although N^1-methylnicotinamide is a prominent member of the niacin derivatives found in urine after tryptophan administration.

Quinolinic acid, on the other hand, which accounts for a large fraction of the niacin derivatives found in urine after the administration of niacin precursors such as 3-hydroxyanthranilic acid (Henderson and Hirsch, '49c) and tryptophan (Henderson et al., '49e), has been shown to have a small but significant role in correcting niacin deficiency in the rat (Henderson, '49b). The present study confirms this slight activity of quinolinic acid, although the degree of activity is somewhat variable, depending upon circumstances. When, for example, quinolinic acid was incorporated in diet A (sucrose diet) at a level of 2 mg% for the first 5 days of the 10-day test period, no growth response was observed, while a weight increase of 21 gm was observed with the same amount of niacinamide. On the other hand, the intraperitoneal injection of 1 mg of quinolinic acid for 10 days (total dosage, 10 mg) in animals on the dextrin diet (diet C) resulted in a net 10-day growth response of 16 gm (after deducting the growth of the control group). Animals on a comparable diet which were given 1 mg of niacinamide for 5 days (total dosage, 5 mg) exhibited a net growth response of 42 gm. The oral administration of 2 mg of quinolinic acid daily for 10 days (total, 20 mg) to animals on dextrin diet C resulted in

a net growth response of about 8 gm (i.e., 20 gm vs. 7.6 gm for the control group on diet C), whereas animals on diet C which received 0.2 mg of niacinamide orally for 5 days (total dosage, 1 mg) gained a net of 30 gm in the 10-day test period. When the oral dosage level of quinolinic acid was increased to 4 mg per day for 10 days for animals on diet C, a near maximum growth response was produced (not shown in table 2).

On the basis of the present data it would appear that the relative activity ratio of quinolinic acid to niacin is about 50 when the compounds are administered by the oral route. It is extremely difficult to evaluate the relative activity ratio between quinolinic acid and niacin when the two compounds are injected because of the differences in dosage levels and the variations in the growth responses obtained. It was found, however, that quinolinic acid had somewhat greater activity when injected intraperitoneally than when it was given orally or incorporated in the diet.

Studies with N. crassa

One strain of *N. crassa*, Y31881, accumulates in its medium a compound that has slight activity for *Neurospora* strain 39401, which is a niacin-requiring strain. Acid hydrolysis of this accumulation product yields a compound that has been isolated and identified as kynurenine, which is considerably more active for *Neurospora* strain 39401 than the parent compound. The accumulation compound in the medium of strain Y31881 has been isolated and identified as α-N-acetylkynurenine (Yanofsky and Bonner, '50) and arises as the result of the inability of mutant strain Y31881 to convert kynurenine to 3-hydroxykynurenine.

Acetyl kynurenine was therefore tested for its ability to induce growth in the niacin-deficient rat when fed for 5 days at a level of 100 mg% in the diet. Even at this high level, no growth response was observed (see table 2). It will be remembered that as little as 1 mg% of niacin and 10 mg%

of L- or DL-tryptophan in the niacin-deficient diet will result in a maximum growth response.

Quinolinic acid has been tested on certain mutant strains of *Neurospora* which require niacin and found to be inactive (Bonner, '48; Mitchell and Nyc, '48a). Henderson, however ('49b), found that very large levels of quinolinic acid (0.05 mg per milliliter) supported the growth of mutant strain 4540. Both Henderson ('49b) and Bonner and Yanofsky ('49) have isolated and identified quinolinic acid in the culture filtrate from strain 3416 grown on a suboptimum quantity of niacin. In growth experiments performed at a low pH, and after correction for percentage dissociation ($pk_1 = 3$, $pk_2 = 5.0$), Bonner and Yanofsky ('49) found that quinolinic acid was approximately 10% as active as niacin for *Neurospora* mutant strain 4540, and that it had a similar slight activity for mutant strains 39401 and 31881. Since 3-hydroxyanthranilic acid, the probable precursor of quinolinic acid and niacin, is approximately as active as niacin for *Neurospora*, it seems unlikely that quinolinic acid serves as a direct intermediate in the conversion of 3-hydroxyanthranilic acid to niacin in *Neurospora*.

Although quinolinic acid accumulates in the medium of mutant strain 3416 when grown on suboptimum amounts of niacin and the addition of 3-hydroxyanthranilic acid was observed in the present study to result in an increased production of quinolinic acid in the medium, L-tryptophan addition failed to yield increased amounts of quinolinic acid.

Enzyme studies

To demonstrate further that quinolinic acid is formed from the niacin precursors tryptophan and 3-hydroxyanthranilic acid, these respective compounds were incubated with liver tissue from adult rats. The enzyme studies were made by adding 500 µg of the substrate (either tryptophan or 3-hydroxyanthranilic acid) to 5 ml of Krebs-Ringer phosphate buffer at pH 7.2 in 25-ml Erlenmeyer flasks which also con-

tained 400 mg ± 10 mg of fresh liver slices. The flasks were incubated while being shaken for three hours at 37°C., after which they were placed in a boiling water bath for 10 minutes. The contents were then homogenized and assayed for niacin and quinolinic acid by the method of Henderson ('49b). As can be seen in table 3, and as has been reported by Henderson and Ramasarma ('49d), a substantial proportion (about 50%) of 3-hydroxyanthranilic acid is converted to quinolinic acid. The present finding, while in disagreement with the experiments of Hurt et al. ('49), agrees with those of Henderson and Ramasarma ('49d) in that no conversion of DL-tryptophan was detectable by the experimental techniques employed. No explanation can be given at present of the aforementioned disagreement.

TABLE 3

Conversion of niacin precursors to quinolinic acid by liver slices

FLASK NO.	SUBSTRATE ADDED	FRESH WEIGHT OF SLICES	NIACIN PER FLASK	
			No treatment	Acetic acid autoclaved 2 hrs.
		mg	*μg*	*μg*
1	None	402	56.8	60.4
2	500 μg DL-tryptophan	410	55.2	58.8
3	500 μg 3-hydroxyanthranilic acid	395	58.8	294.0

DISCUSSION

The much more favorable growth response obtained with 3-hydroxyanthranilic acid in relieving niacin deficiency in the present study as compared to the original observation by Mitchell et al. ('48b) may be due to the fact that it was given intraperitoneally and not subcutaneously. It might be well to point out again the comparable effective doses of tryptophan, 3-hydroxyanthranilic acid, quinolinic acid and niacin required to correct niacin deficiency in the rat; these are, respectively, 10, 10, 20–40, and 0.2 mg using the type of diet employed in the present experiment. On an equimolecular basis the ac-

tivity scale appears to be as follows: niacin >>>, tryptophan >, 3-hydroxyanthranilic acid >> quinolinic acid.

The fact that the liver is capable of converting a large percentage of 3-hydroxyanthranilic acid to quinolinic acid helps to explain the rather low degree of activity of the former compound. That such a reaction takes place does not at all prove that quinolinic acid serves as a direct intermediate in the conversion of tryptophan to niacin. Indeed,

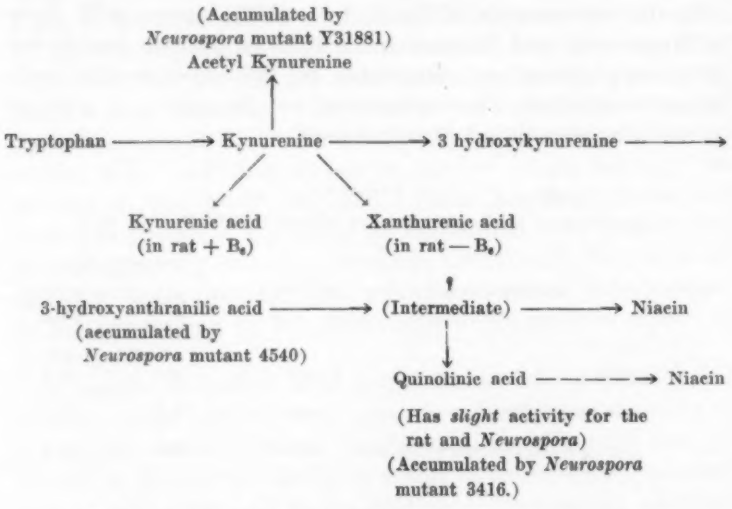

Fig. 1 Tryptophan-niacin relationship in *Neurospora* and rats.

the very fact that 3-hydroxyanthranilic acid exhibits considerably greater growth activity for the rat, yet is proposed to precede quinolinic acid in the tryptophan-to-niacin conversion, could just as well indicate that the conversion of 3-hydroxyanthranilic acid to quinolinic acid is merely a shunting or side reaction, even though quinolinic acid is much closer to niacin in molecular configuration than its precursor, 3-hydroxyanthranilic acid. The possibility that such a side reaction occurs has been suggested on the basis of work with

Neurospora (Bonner and Yanofsky, '49). Although 3-hydroxyanthranilic acid has been shown to serve as a precursor of both niacin and quinolinic acid for *Neurospora*, the fact that quinolinic acid is only slightly active for those *Neurospora* mutants which are known to convert 3-hydroxyanthranilic acid to niacin suggests that quinolinic acid is not a direct niacin precursor. The diagram shown in figure 1 is offered to explain the authors' interpretation of the tryptophan-to-niacin conversion and the role played in it by quinolinic acid.

It is reasonable to think that 3-hydroxyanthranilic acid serves as a precursor of both niacin and quinolinic acid by separate reactions, and that quinolinic acid is shunted out very rapidly as an excretory product since it is present in only very small amounts in liver (Henderson and Ramasarma, '49d).

It should be emphasized that the conversion of quinolinic acid to niacin by *Neurospora* is at best an inefficient reaction which is even more inefficient in the rat. In addition, the fact that the probable precursor of quinolinic acid in both organisms, 3-hydroxyanthranilic acid, is more efficiently converted to niacin suggests that quinolinic acid is not the primary intermediate between 3-hydroxyanthranilic acid and niacin in either organism.

The antipellagragenic and antiblacktongue activity of quinolinic acid has a rather interesting history. Neither Woolley et al. ('38) nor Dann et al. ('40) found it to be active in curing blacktongue in dogs. Dann and co-workers took 0.15 mg per kilogram per day as the preventive dose of nicotinic acid and state that "Under our conditions a dose of quinolinic acid stoichiometrically 25 times greater was ineffective in preventing blacktongue; but a 100-fold greater dose gave complete protection for 156 days." Although Dann observed the almost "theoretical yield of pyrazine monocarboxylic acid" after refluxing quinolinic acid with glacial acetic acid, he did not apply this procedure to his chemical assay of the quinolinic acid used in his test and it is conceivable that the product he used contained somewhat more niacin

than the 0.03% he reported it to contain. Koser et al. ('40) suggest that the reported quinolinic acid activity in treating pellagra and blacktongue is due to the presence of nicotinic acid as an impurity.

The inactivity of kynurenine in promoting growth in the rat is inconsistent with the fact that this compound is considered to be an intermediate in the conversion of tryptophan to niacin. Although Rosen et al. ('47) reported that kynurenine produced no extra urinary N^1-methylnicotinamide in the rat, Kallio and Berg ('49) found that it did lead to increased excretion of this urinary niacin derivative although the production of N^1-methylnicotinamide from kynurenine was less efficient than from L-tryptophan. Kallio and Berg ('49) make the interesting statement that "evidence based on metabolite excretion does not always provide a safe criterion as to relative utility for growth." It has been the authors' contention that growth and correction of niacin deficiency are the most realistic and practical measures of a compound's effectiveness, although such criteria are of limited value in elucidating the individual steps in a metabolic pathway.

SUMMARY

Of numerous niacin derivatives and precursors tested, only niacinamide, 3-hydroxyanthranilic acid, L- and DL-tryptophan and quinolinic acid proved active in correcting niacin deficiency in the rat. Kynurenine, kynurenic acid, anthranilic acid, N-formyl kynurenine, N-formyl anthranilic acid, N^1-methylnicotinamide, 3-cyanopyridine, and acetyl kynurenine proved inactive in this respect.

The incubation of 3-hydroxyanthranilic acid with fresh liver slices resulted in a large percentage conversion to quinolinic acid. Tryptophan was not so converted under similar conditions.

The relative activity of quinolinic acid for rats is so low compared with that of other niacin precursors that it is suggested that it may be produced as a side reaction rather

than as a direct intermediate in the conversion of tryptophan to niacin, in a manner similar to that suggested for *Neurospora*. The basis for this suggestion is discussed.

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